PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

A12

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6:
A61K 31/70, C12N 9/10, 15/12, 15/54

A1

(11) International Publication Number: WO 98/25630

(43) International Publication Date: 18 June 1998 (18.06.98)

(21) International Application Number: PCT/US97/22770

(22) International Filing Date: 12 December 1997 (12.12.97)

(30) Priority Data:
60/032,974 13 December 1996 (13.12.96) US

(71) Applicant: THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK [US/US]; P.O. Box 9, Albany, NY 12201–0009 (US).

(72) Inventors: PERL, Andras; 5200 Silverfox Drive, Jamesville, NY 13210 (US). BANKI, Katalin; 5200 Silverfox Drive, Jamesville, NY 13210 (US).

(74) Agent: BRAMAN, Susan, J.; Jaeckle Fleischmann & Mugel, LLP, 39 State Street, Rochester, NY 14614–1310 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TRANSALDOLASE-MEDIATED REGULATION OF APOPTOSIS

(57) Abstract

Transaldolase (TAL) plays an important role in regulating the sensitivity of cells to apoptosis. Methods which upregulate TAL gene expression, such as by delivery of exogenous TAL–encoding DNA to a cell, or methods which stimulate TAL enzymatic activity, such as induction of phosphorylation through protein kinase C, promote programmed cell death in response to apoptotic signals. Conversely, inhibition of TAL gene expression, such as by delivery of TAL antisense DNA, or the suppression of TAL enzymatic activity, renders the cell resistant to apoptotic signalling. The present invention provides approaches to the treatment of conditions characterized by enhanced apoptosis, for example, neurodegenerative diseases, demyelinating diseases or HIV disease, or conditions in which apoptosis is inappropriately suppressed, for example cancer, certain virus infections and autoimmunity, by the appropriate up– or down–regulation of TAL expression or TAL enzymatic activity.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TRANSALDOLASE-MEDIATED REGULATION OF APOPTOSIS

This invention was funded in part by a Grant from the National Institutes of Health (RO1 DK49221), which provides to the United States government certain rights in this invention.

This application claims priority of U.S. Provisional Patent Application No. 60/032,974, filed December 13, 1996.

FIELD OF THE INVENTION

The invention in the fields of medicine and molecular biology relates to the regulation of apoptosis by overexpression or suppressed expression of human transaldolase (TAL) in cells. DNA encoding TAL or DNA antisense to TAL DNA or small molecules which modulate TAL enzymatic activity by influencing the pentose phosphate pathway (PPP) are useful in treating a disease or disorder which is associated with dysregulation of normal apoptotic signalling and processes.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced, some by roman numerals in parenthesis. Full citations for each of these publications are provided within the text of the application or, for those referred to by roman numerals, at the end of the Detailed Description. The disclosures of each of these publications in their entireties are hereby incorporated by reference in this application.

Apoptosis is a fundamental form of programmed cell death (PCD) which is indispensable for normal development and maintenance of homeostasis within multicellular organisms (1). Defects in apoptosis may underlie the etiology of neurodegenerative diseases, cancer, autoimmune diseases, and the acquired immune deficiency syndrome (3).

Although reactive oxygen intermediates (ROIs) have long been considered as toxic by-products of aerobic

existence, evidence is now accumulating that controlled levels of ROIs modulate cellular function and are necessary for signal-transduction pathways, including those mediating apoptosis (4-7). Many of the chemical and physical stimuli which elicit PCD generate ROIs such as H_2O_2 and OH^- (8). Low doses of H_2O_2 induce apoptosis in a variety of cell types, thus establishing oxidative stress as a mediator of apoptosis (7). The ability of scavengers of ROIs to inhibit apoptosis support this hypothesis (9). Examples of such scavengers include N-acetylcysteine (NAC), a precursor of glutathione (GSH) (7,8) and free radical spin traps such as 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3,5,5-tetramethyl-1-pyrroline-1-oxide (TMPO). Bcl-2, the prototype of a novel family of protooncogenes which inhibit apoptosis when induced by any of a number of diverse stimuli, was recently shown to have antioxidant behavior (5,6). However, apoptosis and bcl-2 protection were demonstrated in very low oxygen pressure, suggesting that ROIs may not be an absolute requirement for apoptosis (10).

A normal reducing atmosphere required for cellular integrity is provided by reduced glutathione (GSH), which protects cells from damage by ROIs (11). Decreased intracellular GSH levels in HIV-infected T cells suggest a role for oxidative stress in HIV-induced cell death (Buhl, R. *et al.*, (1989) *Lancet II*, 1294-1298; Staal, F.J.T. *et al.*, (1992) *Lancet 339*:909-912). Signaling through the APO-1/Fas/CD 95 antigen and the structurally related cell surface receptor for tumor necrosis factor (TNF) has been implicated in accelerating HIV-induced apoptosis.

Synthesis of GSH from its oxidized form, GSSG, is completely dependent on NADPH produced by the pentose phosphate pathway (PPP)(11)(See Figure 11). In fact, a fundamental function of PPP is to maintain glutathione in a reduced state and thus provide protection of sulfhydryl groups and cellular integrity from emerging oxygen

radicals. PPP comprises two separate branches: the oxidative and the nonoxidative. Reactions in the oxidative branch are irreversible, whereas all reactions of the nonoxidative branch are fully reversible. The two branches are functionally connected. The nonoxidative branch can convert ribose 5-phosphate into glucose 6-phosphate for the oxidative branch and thus, indirectly, it can also contribute to generation of NADPH. The rate limiting enzymes for the two branches are different. The oxidative phase is primarily dependent on glucose-6-phosphate dehydrogenase (G6PD; 12). Control of the nonoxidative branch, between transaldolase and transketolase, is less well established based on knowledge of the enzyme kinetics. It was suggested that, based on tissue-specific variations in enzymatic activities, TAL may be a rate-limiting enzyme of the nonoxidative branch of the PPP (12,13,14).

TAL catalyzes the transfer of a 3-carbon fragment, corresponding to dihydroxyacetone, to D-glyceraldehyde 3-phosphate, D-erythrose 4-phosphate, and a variety of other acceptor aldehydes, including nonphosphorylated trioses and tetroses. Enzymatic activity of TAL is regulated in a tissue-specific (13,14) and developmentally-specific manner (15). In the brain, TAL is expressed selectively in oligodendrocytes at high levels (16). This is particularly interesting because myelin sheaths are formed by oligodendrocytes and lesions in the most common demyelinating disease of the CNS, multiple sclerosis (MS), are characterized by a progressive loss of oligodendrocytes and demyelination. Oligodendrocytes are exquisitely sensitive to damage by ROI, such as nitric oxide and tumor necrosis factor-α (TNFα) released by activated macrophages and astrocytes (17).

The pentose phosphate pathway (PPP) fulfills two essential functions in cellular metabolism: (1) the formation of pentose phosphates for synthesis of

nucleotides and nucleic acids; and (2) the generation of NADPH for biosynthetic reactions and for maintenance of GSH at a reduced state in which GSH protects sulfhydryl groups and cellular integrity from oxygen radicals. A number of different approaches have been applied to delineate the mechanism by which PPP is controlled: (a) identification of the rate-limiting enzymes; (b) comparison of mass action ratios with the equilibrium constant and steady-state concentrations with actual flux of intermediates for each enzyme; and (c) identification of enzymes under hormonal or environmental control (12).

Finding of a unifying approach has been complicated by the fact that the PPP comprises two separate branches, the oxidative and nonoxidative branches.

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

The present invention identifies for the first time a pivotal role for TAL in regulating the entire PPP and provides a molecular biological and pharmacological basis for modulating TAL expression and activity which is useful in the treatment of a number of diseases characterized by dysfunctional apoptosis.

The present inventors discovered that levels of TAL expression can determine susceptibility to apoptosis signals. Overexpression of TAL in human T cells was accompanied by a decrease in G6PD and 6-phosphogluconate dehydrogenase (6PGD) activities, a concomitant depletion in NADPH and GSH levels, and increased sensitivity to apoptosis provoked by serum deprivation, H_2O_2, nitric oxide (NO), TNFα, anti-Fas monoclonal antibody and

infection by human immunodeficiency virus (HIV-1). In contrast, suppression of TAL activity increased G6PD and 6PGD activities, augmented GSH levels, and inhibited apoptosis. Thus, susceptibility to apoptosis is subject to regulation by TAL through control of the balance between the two branches of the PPP and its overall output as measured by NADPH and GSH production.

The present invention provides a method to promote apoptosis in a cell comprising providing, either *in vitro* or *in vivo*, to the cell a recombinant TAL DNA molecule in the form of a vector which transforms the cells and which, when expressed, results in overexpression of TAL and stimulation of apoptosis either directly or in response to an apoptotic signal.

Also provided is a method to suppress apoptosis in a cell comprising providing, either *in vitro* or *in vivo*, to the cell a recombinant DNA molecule which encodes an RNA molecule that is antisense to all or part of TAL mRNA and which is capable, when expressed, of suppressing TAL expression in the cell. Such suppression of TAL expression results in suppression of apoptosis in response to an apoptotic signal.

In another embodiment, the invention is directed to a method of stimulating TAL enzymatic activity in a cell by providing to the cell an agent which is a substrate or product of the PPP or which influences the available level of a substrate or product of the PPP such that TAL enzymatic activity is stimulated. Such stimulation results in stimulation or enhancement of apoptosis in the cell either directly or in response to an apoptotic signal.

In yet another embodiment, the invention is directed to a method of inhibiting TAL enzymatic activity in a cell by providing to the cell an agent which is a substrate or product of the PPP or which influences the available level of a substrate or product of the PPP such that TAL enzymatic activity is inhibited. Such

inhibition results in inhibition or suppression of apoptosis in the cell in response to an apoptotic signal.

Such methods are useful in treating a disease or condition characterized by abnormally high levels of, or susceptibility to, apoptosis, or, in contrast, abnormally low levels of, or resistance to, apoptosis. Such diseases are described below.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Figure 1 presents a Western blot analysis of TAL protein levels in cell lines stably transfected with TAL expression vectors oriented in the sense (L26-3/4) and antisense directions (L18-3/1), in comparison to untransfected Jurkat cells (control). 40 μg of protein lysate was loaded in each lane. The TAL protein (38 kDa) was detected with rabbit antibody 169 while actin (42 kDa) was visualized with mouse monoclonal antibody C4. Level of TAL expression relative to actin content was determined by scanning densitometry. TAL/actin ratio in control Jurkat cells was considered as 100%. TAL expression was reduced by 25% in L18-3/1 cells and increased 2.6-fold in L26-3/4 cells;

Figure 2 is a set of graphs showing the rate of apoptotic cell death induced by serum withdrawal, 100 μM H_2O_2, 5 mM SNP, 20 ng/ml TNF-α, and 50 ng/ml anti-Fas monoclonal antibody in L26-3/4, L18-3/1, and control Jurkat cells. Results are means $\pm$ SEM of four independent experiments.

Figure 3 shows the time-course of DNA ladder formation in L26-3/4, L18-3/1, and control Jurkat cells during Fas-induced apoptosis. DNA was extracted at the indicated timepoints after treatment with 50 ng/ml anti-Fas antibody and electrophoresed in a 2% agarose gel.

Apoptotic cell death, as evidenced by DNA fragmentation into 180-200 bp bands, was accelerated in L26-3/4 cells and suppressed in L18-3/1 cells in comparison to control Jurkat cells; mw, 123 bp molecular weight ladder;

Figure 4 presents a flow cytometric analysis of Fas-induced apoptosis in L18-3/1, L26-3/4, and control Jurkat cells. After incubation for 16 h with 50 ng/ml anti-Fas monoclonal antibody, control and Fas-stimulated cells were stained with PI and DNA content was measured by red fluorescence (FL2-R). Apoptotic cells appear as a broad hypodiploid DNA peak preceding the narrow peak of diploid DNA from viable cells. Percentage of hypodiploid cells is indicated for each sample. Data are representative of three independent experiments;

Figure 5 shows results of flow cytometric detection of intracellular ROI in Jurkat cells by DCFH-DA and DHR. Cells were treated with 100 μM H_2O_2 or 50 ng/ml anti-Fas monoclonal antibody for 16 hr and labeled with 0.1 μM DCFH-DA or DHR before flow cytometry. Shaded curves correspond to control cells, while open curves represent H_2O_2- or Fas-stimulated cells. X axis shows cell number; Y axis indicates log fluorescence intensity;

Figure 6 shows the time-course of ROI formation and cell death in Jurkat cells. ROI production was measured in log fluorescence intensity by flow cytometry after labeling with 0.1 μM DHR for 2 min. % survival was determined by trypan blue exclusion;

Figure 7 is a graph showing effect of N-acetylcysteine (NAC), buthionine sulfoximine (BSO), and DMPO on GSH levels and TAL and G6PD activities in Jurkat cells;

Figure 8 is a graph showing the effect of antioxidants (NAC, NDGA, DEF, Amytal), prooxidant (BSO), and spin traps (DMPO, TMPO) on Fas-induced cell death. Jurkat cells were treated with 3 mM NAC, 1 mM BSO, 20 mM DMPO, 10 mM TMPO, 100 μM desferrioxamine (DEF), 20 μM nordihydroguaiaretic acid (NDGA), and 400 μM Amytal for

90 min and incubated with 50 ng/ml anti-Fas monoclonal antibody. Cell survival was quantified by trypan blue exclusion. Data represent mean ± SE of three independent experiments;

Figure 9 is a graph showing the rate of apoptosis (cell survival) of transfected Jurkat cells infected with HIV. The groups are as follows:

_____ Jurkat-tat cells (mock transfected)
..... Jurkat control cells
---------- Jurkat L26-3/4 (transfected with TAL DNA)
•••••••••••• Jurkat L18-3/1 (transfected with TAL antisense DNA);

Figure 10 is a graph showing the effect of phosphorylation of TAL by protein kinase C (PKC). The ordinate shows the enzymatic activity of 50 ng of affinity-purified human recombinant TAL (rTAL-H) measured in the forward (TAL-FOR) and reverse (TAL-REV) reactions. The control group is untreated rTAL-H. In the PKC group, rTAL-H was phosphorylated with 25 ng of protein kinase C (Promega, Madison, WI) in the presence of 4 mM Tris pH 7.4, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 0.1 mM sodium metavanadate, 0.1 mM NaF, 0.1 mM ATP, 5 μl of 10x phosphatidyl serine (GIBCO-BRL) for 5 min at 30°C. The PKC + INH reaction represents activity of rTAL-H following pretreatment with PKC in the presence of 20 μM PKC 19-36 inhibitor peptide (GIBCO-BRL); and

Figure 11 is a schematic description of the pentose phosphate pathway and its byproducts showing the enzymes involved in both branches, the sugar phosphate substrates and products, and the generation of NADPH and reduced glutathione.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides approaches to treating a number of diseases or conditions characterized by altered apoptotic behavior or responsiveness to normal apoptotic signals. Table 1 lists a number of conditions

classified in accordance with the apoptosis dysregulation.

Uses of the Invention

Subjects having a disease or condition associated with either increased or suppressed apoptosis can be treated using the methods of the present invention. Cells of the subject can be transfected *in vitro* or *in vivo* with a vector comprising (a) TAL-encoding DNA or (b) DNA encoding mRNA which is antisense to TAL mRNA, which, as exemplified below, results (respectively) in either overexpression of TAL and enhanced apoptosis or inhibition of TAL expression and suppressed apoptosis. As used herein, the above vectors are referred to collectively as TAL DNA construct and more particularly, as either a TAL-encoding DNA construct or a TAL antisense construct.

Thus the present invention is used to promote apoptosis by overexpressing TAL or by upregulating TAL activity in a cell for the treatment of diseases and conditions which include those listed in the left column of Table 1 and any other conditions presently known or later discovered which are characterized by abnormally low apoptosis. The preferred targets for overexpression of TAL or upregulation of TAL activity are various tumors and cancer, wherein normal regulation of cell growth is lost in part due to lack of normal apoptosis. Vectors useful for transfecting cells for TAL overexpression are described and exemplified below.

In another embodiment, a TAL antisense construct is used to deliver DNA which encodes an mRNA molecule or appropriate mRNA fragments which are capable of inhibiting endogenous cellular TAL gene expression leading to the suppression of apoptosis. Alternatively, suppression of apoptosis is achieved by suppression of TAL enzymatic activity in the cell. Such suppression of apoptosis is desirable for the treatment of diseases and conditions which include those listed in the right column

of Table 1. Preferred targets of this embodiment include HIV disease, and neurodegenerative and demyelinating diseases such as multiple sclerosis (MS). In the case of MS, the present inventors have shown that TAL levels are elevated in oligodendrocytes leading to their demise and loss of their myelin production. Similarly, neurodegenerative diseases, in particular Alzheimers disease, are associated with enhanced cell death by apoptosis. Interference with the apoptotic program by expression of TAL antisense DNA would prevent or at least attenuate the loss of neurons and the resultant cognitive dysfunction. The role of apoptotic pathways in neuronal cell death are reviewed in Schwartz, L.E. *et al.* (1996) *Trends in Neurosci. 19*:555-562.

Hence, in one embodiment, the invention comprises parenteral administration of a single or multiple doses of the TAL DNA construct in the form of a retroviral vector or naked DNA preparation (see below) to a mammalian subject, preferably a human. An effective amount of the DNA preparation is a function of the nature of the vector, the patient and his clinical status, and can vary from about 0.01 mg/kg body weight to about 1 g/kg body weight. A subject can be given this amount in a single dose or in multiple repeated doses.

Alternatively, the TAL DNA construct is used to transfer the TAL gene or antisense sequence to cells of the subject *ex vivo* followed by administration of the transformed or transfected cells into the subject. Cells expressing the TAL DNA construct are preferably administered at a dose between about 10^6 and 10^{10} cells on one or several occasions.

In general, the dose used in the present invention will in part be dependent upon the health and weight of the recipient, the existence of other concurrent treatment, if any, frequency of treatment, and the nature of the effect desired, for example, eradication of a tumor or treatment of autoimmunity.

The TAL DNA construct may be advantageously utilized in combination with other therapeutic agents known to be useful in the treatment of the particular disease or condition being treated.

Administration of DNA or a vector or other vehicle containing the DNA or cells, as in the gene therapy embodiments described herein, is performed using any of a number of routes, depending on the location of the cells being targeted. Thus, administration may be by any parenteral route, including but not limited to intramuscular, subcutaneous or transdermal, intravenous (including into the portal circulation), intrathecal, intraperitoneal, intragastric and by inhalation or instillation into the lungs. Oral administration of certain of the delivery vehicles disclosed herein is also known in the art. A preferred route is by iv injection or infusion.

The present invention may be directed to the treatment of disorders associated with increased cell survival. Diseases characterized by the accumulation of cells include cancer, autoimmune diseases, and certain viral illnesses as well as benign hyperplasia and vascular restenosis. Cell accumulation can result from either increased proliferation or the failure of cells to undergo apoptosis in response to appropriate stimuli. Alterations in the control of cell survival are important in the pathogenesis of these so-called proliferative disorders.

Cells from a wide variety of human malignancies have a decreased ability to undergo apoptosis in response to at least some physiologic stimuli (Hoffman, B. *et al.*, *Oncogene 9*:1807 (1994)), in particular in metastatic tumors. The molecular bases for the increased resistance of tumor cells to undergo apoptosis is currently under active investigation. *bcl2* is a member of a family of genes that can control the apoptotic threshold of a cell (Boise, L.H. *et al.*, *Cell 74*:597 (1993); E. Y. Lin *et*

al., J. Immunol. 151:1979 (1993); K. M. Kozopas *et al., Proc. Natl. Acad. Sci. USA 90*: 3516 (1993); Z. N. Oltvai *et al., Cell 74*:609 (1993)). Overexpression of *bcl2* or of its homologues confers resistance to cell death caused by chemotherapeutic agents (T. Ohmon *et al., Biochem. Biophys. Res. Commun. 192*:30 (1993)). The primary mechanism by which most chemotherapeutic agents induce cell death is by altering cell physiology leading to the induction of apoptosis. Cell death in response to DNA damage by these or other agents typically results from apoptosis. Thus, the use of a TAL-encoding DNA construct to overexpress TAL and promote or induce apoptosis, as disclosed herein, provides a novel form of chemotherapeutic intervention against cancer.

The role of apoptotic signalling in autoimmune diseases is not clear at the moment, and some controversy exists in the art. Proper regulation of cell death is essential for eliminating potentially autoreactive lymphocytes during development and for removing excess cells after the completion of an immune response. Failure to remove autoimmune cells that arise during development or that develop as a result of somatic mutation during an immune response can result in autoimmune disease. The cell surface receptor Fas is considered critical in regulating cell death in lymphocytes (Watanabe-Fukunaga *et al., Nature 356*:314 (1992)). Stimulation of Fas on activated lymphocytes induces apoptosis. *bcl-2* blocks *fas*-mediated cell death (Itoh, N. *et al., J. Immunol. 151*:621-627 (1993)) by inhibiting the action of the cysteine protease interleukin-1β-converting enzyme (ICE). Two forms of hereditary autoimmune disease are attributed to alterations in Fas-mediated apoptosis (Watanabe-Fukunaga, *supra*; T. Suda *et al., Cell 75*:1169 (1993)).

Transformation of cells with TAL-encoding DNA to promote, or prime the cells for, apoptosis and cell death, as described herein, is therefore useful in arresting the progression of autoimmune diseases such as,

for example, rheumatoid arthritis, psoriasis, inflammatory bowel disease, and autoimmune diabetes mellitus, just to name a few.

The present invention is also useful in the treatment of virus infections. The disruption of cell physiology following viral infection can cause an infected cell to undergo apoptosis (B. Levine *et al.*, *Nature 361*:739 (1993)). The suicide of an infected cell or its lysis by specific cytotoxic T lymphocytes are both cellular defense mechanisms to prevent viral propagation. T cells can induce cell death by activating the target cell's endogenous cell death program (D. Kagieta *et al.*, *Science 265*:528 (1994)). Viruses can circumvent such defenses by disrupting the normal regulation of apoptosis in infected cells. Viruses for which apoptosis-blocking proteins have been identified include adenovirus (Rao, L. *et al.*, *Proc. Natl. Acad. Sci. USA 89*:7742 (1992)), Epstein-Barr virus and African swine fever virus (Neilan, J.G. *et al.*, *J. Virol. 67*:4391 (1993)), baculoviruses (Clem, R.J. *et al.*, *Science 254*:1388 (1991)), and poxviruses (Ray, C.A. *et al.*. *Cell 69*:597 (1992)).

Thus, DNA constructs of the present invention can be used in an antiviral therapeutic approach, by introduction into cells infected with virus. This is particularly important for latent viral infection or situations in which viral replication does not lead to cell death. Indeed, the prevention of apoptosis by a virus is important for the establishment of viral latency. For example, EBV establishes a latent infection in B lymphocytes and expresses a gene, *LMP-I*, which specifically upregulates the expression of *bcl2*, providing a survival advantage to latently infected cells (Henderson *et al.*, *Cell 65*:1107 (1991)). Chronic Sindbis virus infection also depends on the host cells' expression of *bcl2* (Levine, B. *et al.*, *Nature 361*:739 (1993)). According to the present invention, TAL-encoding DNA is introduced into chronically infected

cells to overcome the anti-apoptotic action of viral genes, resulting in death of the cells and eradication of the chronic or latent infection.

Apoptosis of virus-infected cells has become recognized as an important pathophysiologic mechanism in HIV disease and AIDS (Gougeon, M-L. *et al.* (1993) *Science.* 268:1269; Ameisen, J-C. (1992) *Immunol. Today.* *13*:388). Furthermore, bystander T cells are at risk for apoptotic death in HIV infected subjects (Finkel, T *et al.*, (1994) *Curr. Opin. Immunol.* *6*:605; Banda, N. *et al.*, (1992) *J. Exp. Med.* *176*:1099; Newell *et al.* (1990) *Nature* *347*:286; Oyaizu, N. *et al.* (1994) *Blood* *84*:2622; Desbarats, J. *et al.* (1996) *Proc. Natl. Acad. Sci. USA* *93*:11014-11018). The present invention can attenuate the apoptosis, primarily of T lymphocytes, caused by HIV infection by blocking expression or enzymatic activity of TAL as described herein.

In contrast to many other cell types, most neurons survive for the life of the organism. A wide variety of neurological diseases are characterized by the gradual loss of specific sets of neurons (Isacson, O., *Trends Neurosci.* *16*:306 (1993); Heintz, N., *Trends Biochem. Sci.* *18*:157 (1993)). Such disorders include Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, spinal muscular atrophy, and various forms of cerebellar degeneration, diseases in which cell death results in specific disorders of movement and central nervous system function. Apoptosis appears to be the mechanism of cell death in such neurodegenerative diseases. Oxidative stress, calcium toxicity, mitochondrial defects, excitatory toxicity, and deficiency of survival factors are proposed pathogenetic determinants (Cow, D.W., *J. Neurobiol.* *23*:1261 (1992); Zivetal, I., *Neuroscl Lett.* *170*:136 (1994)). Each of these pathways predisposes neurons to apoptosis, either *in vitro* or *in vivo*, whereas overexpression of *bcl2* decreases the neurotoxicity of

each of these potential inducers (Zhong, L.T. et *al.*, *Proc. Natl. Acad. Sci U.S.A. 90*:4533 (1993)). Neurotrophic growth factors and the extracellular matrix also alter the apoptotic threshold of neural cells (Arenas, E. *et al.*, *Nature 367*:368 (1994)). Together, these observations suggest that the threshold for cell death is dynamically regulated such that the apoptotic threshold of a cell is determined by the combined effects of external and internal survival factors. According to the present invention, TAL levels contribute to this emerging model.

A form of hereditary ALS results from mutations in the copper-zinc superoxide dismutase gene which renders cells less able to detoxify ROIs. Superoxide-induced death could be specifically inhibited by treatment with survival growth factors or antioxidants (Troy, C.M. *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 91*:6384 (1994)). Retinal degeneration in retinitis pigmentosa results from mutations in any one of three photoreceptor-specific genes: rhodopsin, the β subunit of cyclic guanosine monophosphate phosphodiesterase, and the peripherin gene. All three mutations lead to photoreceptor apoptosis (Chang,, G-O. *et al.* , *Neuron 11*:595 (1993)). Apoptosis may be initiated in response to the accumulation of mutant proteins or as a result of the altered functional properties of the mutant proteins and results suggest that certain neurotrophic and growth factors can enhance photoreceptor survival.

Alzheimer's disease is associated with the progressive accumulation of β-amyloid peptide in plaques. Mutations in the β-amyloid precursor protein are associated with some forms of familial Alzheimer's disease. β-amyloid peptide induces neurons to undergo apoptosis (Loo, D.T. *et al.*, *Proc. Natl. Acad. Sci U.S.A. 90*:7951 (1993)), an effect which can be reversed by antioxidants (C. Behl, C. *et al.*, *Biochem. Biophys. Res. Commun. 186*:944 (1992)).

The spinal muscular atrophies, a group of recessive neurodegenerative disorders of childhood, are characterized by progressive spinal cord motor neuron depletion. One of the genes linked to these disorders, neuronal apoptosis inhibitory protein (NAIP), is homologous to the baculovirus inhibitor of apoptosis protein (IAP) (Roy, N. *et al.*, *Cell 80*:167 (1995)). Mutations in the NAIP gene may therefore result in motor neurons being more susceptible to apoptosis in patients with spinal muscular atrophy. Inhibition of apoptosis using TAL antisense DNA treatment or down-regulation of enzymatic activity, as described herein, are used to treat these disorders.

Thus, the present invention provides methods to inhibit the apoptosis associated with neuronal degeneration in a number of pathogenetic settings by either suppression of the expression of TAL in neurons or by inhibiting neuronal TAL enzymatic activity, as has been described above. The treatments of the present invention may be combined with neuronal growth factors that are know in the art to achieve additive and even synergistic therapeutic effects in neuronal degenerative, as well as in demyelinating diseases and disorders.

Plasmids and Vectors

The DNA molecules of the present invention may be expressed using any appropriate expression vector as is well-known in the art (Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989). More generally, a DNA molecule encoding TAL (or encoding a functional derivative thereof having TAL enzymatic activity) may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, ligation with ap-

propriate ligases, or the synthesis of fragments by the polymerase chain reaction (PCR). Techniques for such manipulations are disclosed by Sambrook, *et al.* (*supra*) and are well known in the art.

To target a particular type of cell, for example tumor cells growing *in vivo*, any of a number of alternate vectors which include the TAL-encoding DNA molecules of the present invention may be selected. Commercially available vectors such as Hook and pCEP4 from Invitrogen may be used.

First, control sequences with tissue specificity for the tissue type of the target cells may be used. Examples of promoters with such specific modes of action include the insulin gene promoter for selective expression in the pancreas or the MMTV or lactalbumin promoter for expression in breast tissue.

For expression of TAL (or other functional derivative) from the plasmids in the target cells, the endogenous translation stop codons may be utilized. If a TAL DNA construct having a C-terminal truncation is used in which the endogenous stop codon is lacking, a stop codon is inserted in the vector just downstream of the cloning site.

For transfection of cells *in vitro* according to the present invention, a selectable marker gene (such as G418-resistance) may be added, either on the same plasmid or by contransfection using a second plasmid such as pSV2neo (Southern, P.J. *et al. J Mol Appl Genet* (1982) *1*:327-341) or the plPB1 plasmid (Biamonti, G. *et al. Nucl Acid Res* (1985) *13*:5547-5561). For transfection of cells with TAL DNA *in vivo*, a selection marker useful *in vivo* is required, for example, the *tk* gene of HSV (see below).

Promoters and Enhancers

A promoter is a region of a DNA or RNA molecule which is capable of binding RNA polymerase and promoting the transcription of an operably linked nucleic acid

sequence. As used herein, a promoter sequence is the sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. Two sequences of a nucleic acid molecule, such as a promoter and a coding sequence, are said to be operably linked when they are linked to each other in a manner which either permits both sequences to be transcribed onto the same RNA transcript, or permits an RNA transcript, begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and a coding sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked coding sequence. In order to be operably linked it is not necessary that two sequences be immediately adjacent to one another.

The promoter sequences of the present invention necessary for expression of the DNA of the invention must be functional in mammalian cells, and may be either eukaryotic or viral promoters. Suitable promoters are inducible, repressible or constitutive. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al.*, *Cell 41*:521 (1985)) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, C.M., *Proc. Natl. Acad. Sci. USA 79*:6777 (1982)). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)) inducible by heavy metal salts such as $CdCl_2$; the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature 290*:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A.,

et al., *Proc. Natl. Acad. Sci. (USA)* *81*:5951-5955 (1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al.*, *Nature* (1986) *231*:699; Fields *et al.*, *Nature* (1989) 340:245; Jones, *Cell* (1990) *61*:9; Lewin, Cell (1990) *61*:1161; Ptashne *et al.*, *Nature* (1990) *346*:329; Adams *et al.*, *Cell* (1993) 72:306.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in Roy-Burman *et al.*, U.S. Patent No. 5,112,767. For a general discussion of enhancers and their actions in transcription, see, Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990), pp. 552-576. Particularly useful are retroviral enhancers (*e.g.*, viral LTR). The enhancer is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency on the TAL encoding DNA molecule of the present invention.

Inducible Systems for TAL Overexpression or Suppression

The utility of inducible (in contrast to constitutive) systems for inducing apoptosis is exemplified by the Tet-Off and Tet-On system, originally described by Gossen, M. *et al.*, *Proc. Natl. Acad. Sci. USA* *89*:5547-5551 (1992)). Vectors for such a controllable system are now commercially available from

CloneTech. This approach is based on the use of control elements of the tetracycline-resistance operon encoded in Tn10 of *E. coli*. The *tet* repressor is fused with the activating domain of Herpes simplex virus VP16 to generate a tetracycline-controlled transactivator. Such a transactivator is used to stimulate transcription from a promoter sequence, such as the human CMV promoter IE. This is a repressible system in contrast to the estrogen-inducible system described below. A gene controlled by a promoter acting under the influence of the tetracycline-controlled transactivator can be constitutively expressed and turned off by using an effective concentration of tetracycline. Such a system can regulate a gene over about five orders of magnitude. The tetracycline-repressible system functions *in vivo* in mammals, where tetracycline administration via the diet is used to keep the expression of the inducible gene off. Tetracycline analogs which cross the blood-brain barrier can be used if gene activity is desired in the brain.

According to the present invention, the TAL DNA is placed under the control of a promoter subject to regulation by a tetracycline-controlled transactivator. Such a construct (in a single vector or preferably two vector form) is delivered into target cells such as tumor cells growing *in vivo*. To kill the tumor cells, tetracycline is withheld so that the TAL is overexpressed. To prevent the action of the TAL DNA locally, tetracycline or an active congener of tetracycline is administered locally to the cells transfected with the constructs. Effective systemic doses (oral or parenteral) of tetracycline are in the range of about 0.1 mg to 1 g per day. In a preferred embodiment, the tetracycline-repressible construct is introduced into selected cells, such as cells of a particular tumor. The transactivator is maintained in the off position using tetracycline until the desirable localization can be demonstrated. At that time,

tetracycline is withheld, stimulating expression of TAL leading to more rapid apoptotic death of the transfected cells.

Other inducible promoters well-known in the art can be used to produce analogous inducible systems for expression of the DNA molecules according to the present invention and for the induction of apoptosis *in vitro* or *in vivo*. Thus, one means for inducing apoptosis in a controllable manner is to use an TAL DNA construct in combination with inducible or repressible control elements such as an estrogen-inducible system (Braselmann, S. *et al. Proc Natl Acad Sci USA* (1993) *90*:1657-1661). In such a system, the TAL DNA or portion thereof encoding an effective TAL enzyme (or a TAL antisense construct) is controlled by a GAL4-responsive promoter which is transactivated in the presence of 17-β estradiol by the GAL-ER-Vp16 transcription factor, a fusion protein consisting of the DNA-binding domain of GAL4, the estrogen-binding domain of the estrogen receptor and the transactivation domain of Vp16 (of Herpes simplex virus). For induction of expression of the TAL DNA molecules in an estrogen-inducible system in an animal, local or systemic treatment with estrogen is required. An effective dose of an estrogen is a dose which would trigger the expression of the TAL-encoding DNA to induce apoptosis of cells such as tumor cells. Such doses can be ascertained by one skilled in the art. Preferably, doses in the range of about 0.05 to 100 mg/kg of an estrogen are used in a single dose or in multiple doses over a period of about one week to about 2 months, or even longer. Forms and preparations of estrogen and their usage in animals, particularly in humans, are well-known in the art (Gilman, A.G. *et al.*, *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 7th Ed., MacMillan Publ. Co., New York, 1985). Estrogen analogs which are capable of specifically activating the

exogenous transactivator while having fewer biological effects and side effects are preferred.

Most known approaches to gene therapy for the treatment of neoplastic or other cell proliferative diseases are not well localized. Ionizing radiation has been used to activate the transcription of exogenous genes that encode a cytotoxic protein such as TNFα (Weichselbaum, R.R. *et al.*, *Int. J. Radiation Oncology Biol. Phys. 24*:565-567 (1992)). This may be accomplished through the use of radiation-responsive elements distal to the transcription start site of such genes. See, for example, Hallahan, D. *et al. Proc. Natl. Acad. Sci. USA 88*:2152-2160 (1991); Datta, R. *et al., Proc. Natl. Acad. Sci. USA 89*:10149-10153 (1992); Weichselbaum, R.R. *et al., Int. J. Radiation Oncol. Biol. Phys. 24*:565-567 (1992); Hallahan, D.E. *et al. J. Biol. Chem. 268*:4903-4907 (1993); Weichselbaum, R.R. *et al., Intl. J. Radiation Oncology Biol. Phys. 30*:229-234 (1994); Hallahan, D.E. *et al. Nature Med. 1*:786-791 (1995). Thus, the present invention provides methods for the spatial and temporal control of gene therapy using a TAL DNA construct with such a radiation-inducible promoter to activate TAL and thereby induce apoptosis. This method for treating neoplastic disease also takes advantage of the direct anti-tumor effects of the radiation itself, resulting in an additive or synergistic interaction between the cytotoxic action of the TAL overexpression and radiation. For treating tumor metastases, it is possible to radiate metastases in one site or in multiple organs such that radiation will preferentially activate TAL overexpression in the irradiated volume. This approach is also applicable to local disease where radiosensitizers can be used in combination with irradiation for direct cytotoxicity and/or activation of transcription of TAL with subsequent apoptotic tumor cell death. The present invention has advantages over the system using TNF described by Hallahan, Weichselbaum and

colleagues for sparing surrounding tissue because TAL activation is intracellular. Only cells carrying the construct will be killed whereas TNFα is activated, diffuses out and acts regionally (and may even reach more distant sites where it could exert undesired toxic effects).

The TAL-encoding DNA is placed in a vector under control of a radiation-inducible promoter. In one embodiment, a genetic construct with a VP-16 DNA sequence that encodes a known powerful transactivating protein attached to the DNA coding sequence derived from the DNA binding domain or the Lac repressor is inserted downstream of Cis-acting elements which bind radiation-inducible proteins. These constructs are useful in amplifying radiation-induced signals. This construct would be cotransfected with the plasmid containing multiple DNA binding sites for the Lac repressor protein cloned upstream of genes which when activated alter the phenotypic response of tumors to radiation. In a preferred embodiment, TAL DNA or an active polynucleotide fragment thereof is recombined with a replication-deficient adenovirus type 5 (McGrory, *et al. Virology 163*:614-617 (1988)) to yield a vector designated Ad.Egr-TAL (similar to the Ad.Egr-TNF vector made by GenVec, Rockville, MD, and described in Hallahan, D.E. et al., 1995, *supra*). This vector employs the CCA(A+T rich)$_6$GG elements (known as CArG elements) within the 5'-untranslated region of the early growth response (Egr-1) promoter 425 bp upstream from the transcription start site (Datta *et al.*, *supra*). A control region containing the 6 CArG elements of the promoter/enhancer region of the Egr-1 gene is ligated upstream of the TAL-encoding DNA. These control elements are known to be inducible in several types of human tumor cells. Other DNA sequences that activate transcription after X-irradiation and which may be used in the present method include AP-1 (Hallahan

et al., 1993, *supra*) and the NκRB binding sequence (Brach, M. *et al.*, *J. Clin. Invest.* *88*:691-695 (1991)).

Tumor cells (or other cells to be treated according to the invention) are injected with or otherwise administered, on one or on multiple occasions, about 2 x 10^8 PFU of AD5.Egr-TAL. At an appropriate time thereafter, ranging from several hours to several days, or even weeks, the target tissue, typically tumor, is irradiated with an effective dose of X-irradiation. The preferred radiation regimen can be determined readily by the skilled artisan using conventional clinical judgment. The dose and time course are a function of the nature and extent of disease, the particular promoter used and its responsiveness, and the treatment approach (*e.g.*, whether the radiation is being relied upon to kill cells directly, to induce apoptosis through TAL activation or both). In one embodiment, 5 Gy X-irradiation are given four times per week for a total of 50 Gy, for example from a Maxitron generator (1.88 Gy/min).

An advantage of the foregoing method is that transcriptional activation of a promoter is controlled by ionizing radiation within a specific body volume and for a chosen period of time. This achieves both spatial and temporal regulation of TAL transcription and overexpression, allowing apoptosis to be induced at a desired time and in a desired volume of cells or tissue. Such regional radiation exposure avoids the possibility of a broader or systemic apoptosis-inducing effect. Thus, cells which have incorporated and are capable of expressing the TAL gene but are not the intended targets of apoptosis induction are spared by excluding them from the volume being irradiated. In this manner, the radiation can be used for spatial control of TAL-assisted cell killing.

Antisense Oligonucleotides and Polynucleotides

The methods of the present invention use a poly- or oligonucleotide which comprises an antisense sequence or

encodes an antisense RNA which is antisense to an endogenous DNA or mRNA TAL sequence.

The present invention uses antisense oligonucleotides and polynucleotides complementary to the gene or genes encoding TAL in a eukaryotic cell, preferably a human cell. Such antisense oligonucleotides should be at least about six nucleotides in length to provide minimal specificity of hybridization, and may be complementary to one strand of DNA or to mRNA encoding TAL (or to a portion thereof), or to flanking sequences in genomic DNA which are involved in regulating TAL gene expression. The antisense oligonucleotide may be as large as about 100 nucleotides, and may extend in length up to and beyond the full coding sequence for which it is antisense. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded.

The action of the antisense nucleotide may result in specific alteration, primarily inhibition, of TAL gene expression in cells. For a general discussion of antisense, see: Albers, B., *et al.*, *MOLECULAR BIOLOGY OF THE CELL*, 2nd Ed., Garland Publishing, Inc., New York, NY (1989), in particular, pages 195-196.

The antisense oligonucleotide may be complementary to any portion of the TAL encoding sequence. In one embodiment, the antisense oligonucleotide may be between about 6 and 100 nucleotides, and may be complementary to the initiation ATG codon and an upstream, non-coding translation initiation site of the TAL sequence. For example, antisense nucleotides complementary primarily for non-coding sequence are known to be effective inhibitors of the expression of genes encoding transcription factors (Branch, M.A., 1993 *Molec. Cell. Biol. 13*:4284-4290).

Preferred antisense oligonucleotides are complementary to a portion of the mRNA encoding TAL. For instance, it is expected that by introducing a full

length cDNA clone gene in an antisense orientation, successful alteration of gene expression will be most probable. Naturally, introduction of partial sequences, targeting to specific regions of the gene and the like can be effective as well. An example of a preferred antisense oligonucleotide is a 50mer which is antisense to 50 nucleotides in the 5′ half of an RNA transcript of a TAL-encoding cDNA, more preferably any stretch of 50 nucleotides in the first 500 nucleotides of the 5′ part of the RNA transcript. For example, the antisense oligonucleotide can be antisense to nucleotides 1-50, 2-51, 3-52, 4-53,5-54, *etc.*, of the RNA transcript. Alternatively, the antisense oligonucleotide can be shorter, for example a 30-mer, and be antisense to any 30 nucleotide stretch of the RNA transcript, preferably in the first 500 5′ nucleotides.

As is readily discernible by one of ordinary skill in the art, the minimal amount of homology required by the present invention is that sufficient to result in sufficient complementarity to provide recognition of the specific target RNA and inhibition or reduction of its translation or function while not affecting function of other mRNA molecules and the expression of other genes. While the antisense oligonucleotides of the invention comprise sequences complementary to at least a portion of an RNA transcript of TAL, absolute complementarity, although preferred, may not be required. A sequence "complementary to at least a portion of" another sequence, as referred to herein, may have sufficient complementarity to be able to hybridize with that other sequence *in vivo*, perhaps forming a stable duplex. Naturally, the ability to hybridize may depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with the TAL target sequence it may contain and still form a stable duplex. One skilled in the art can ascertain a

tolerable degree of mismatch by use of standard procedures to determine the melting temperature of the hybridized complex as discussed above.

The antisense RNA oligonucleotides generated intracellularly by transcription can be transcribed from exogenously introduced nucleic acid sequences. Thus, antisense RNA may be delivered to a cell by transformation or transfection or infection with a vector, such as a plasmid or a virus, into which is incorporated (a) DNA encoding the antisense RNA; and (b) the appropriate regulatory sequences, including a promoter, to express the antisense RNA in a target host cell. Within the cell the exogenous DNA or a portion thereof may be transcribed, producing an antisense RNA of the invention. Vectors can be plasmid, viral, or others known in the art which are used for replication and expression in eukaryotic, preferably mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in eukaryotic, preferably mammalian, cells. Such promoters can be inducible or preferably are constitutive as described above. Such a vector, preferably a plasmid, becomes chromosomally integrated such that it can be transcribed to produce the desired antisense RNA. Such plasmid or viral vectors can be constructed by recombinant DNA technology methods that are standard in the art. An oligonucleotide, between about 6 and about 100 bases in length and complementary to the target sequence of TAL may be prepared by chemical synthesis from mononucleotides or shorter oligonucleotides, or produced by recombinant means.

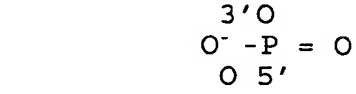
An oligonucleotide, between about 6 and about 100 bases in length and complementary to the target sequence of TAL may be synthesized chemically from natural mononucleosides or, alternatively, from mononucleosides having substitutions at the non-bridging phosphorous

bound oxygens. Alternatively, the oligonucleotide may be produced by recombinant means.

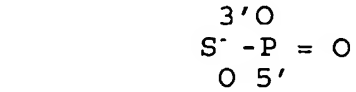
A preferred mononucleoside analogue is a methylphosphonate analogue of the naturally occurring mononucleosides. More generally, the mononucleoside analogue is any analogue whose use results in an oligonucleotide which has improved diffusion through cell membranes or increased resistance to nuclease digestion within the body of a subject (Miller, P.S. *et al.*, Biochemistry 20:1874-1880 (1981)). Such nucleoside analogues are well-known in the art, and their use in the inhibition of gene expression has been disclosed. See, for example, Miller, P.S. *et al.*, *supra*.

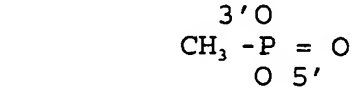
The antisense oligonucleotide molecule of the present invention may be a native DNA or RNA molecule or an analogue of DNA or RNA. The present invention is not limited to use of any particular DNA or RNA analogue, provided it is capable of adequate hybridization to the complementary genomic DNA (or mRNA) of TAL, has adequate resistance to nucleases, and adequate bioavailability and cell uptake. DNA or RNA may be made more resistant to *in vivo* degradation by enzymes such as nucleases, by modifying internucleoside linkages (*e.g.*, methylphosphonates or phosphorothioates) or by incorporating modified nucleosides (*e.g.*, 2'-O-methylribose or 1'-α-anomers).

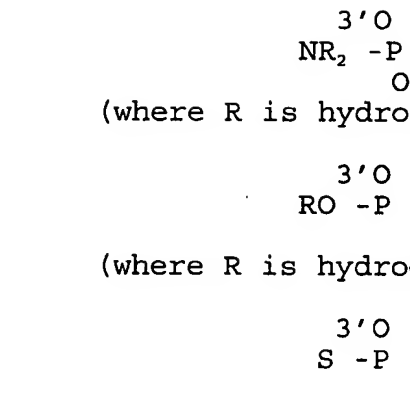
The naturally occurring linkage is



Alternative linkages include the following:







(where R is hydrogen and/or alkyl)

(where R is hydrogen or alkyl)

It is also possible to replace the 3'O-P-O5' with other linkages such as

3'O-CH_2C(O)-O5', 3'O-C(O)-NH5', and 3'C-CH_2CH_2S-C5'.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-carboxymethylaminomethyl-ω-thiouridine, 5-carboxymethyl-aminomethyl uracil, dihydrouracil, β-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyamino-methyl-2-thiouracil, β-D-mannosylqueosine, 5-methoxy-carboxymethyluracil, 5-methoxyuracil-2-methylthio-N6-iso-pentenyladenine, uracil-5-oxyacetic acid, butoxosine, pseudouracil, queosine, 2-thio-cytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-t-oxyacetic acid, 5-methyl-2-thiouracil, 3(3-amino-3-N-2-carboxypropyl) uracil and 2,6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the

group including, but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphoridothioate, a phosphoramidothioate, a phosphoramidate, a phosphordiimidate, a methylsphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide which forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier *et al.*, 1987, *Nucl. Acids Res.* *15*:6625-6641).

The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, a hybridization triggered cross-linking agent, a transport agent, a hybridization-triggered cleavage agent, *etc.*, all of which are well-known in the art.

Oligonucleotides of this invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, *etc.*). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al.*, 1988 *Nucl. Acids Res. 16*:3209, methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, 1988 *Proc. Natl. Acad. Sci. U.S.A. 85*:7448-7451), *etc.*

Basic procedures for constructing recombinant DNA and RNA molecules in accordance with the present invention are disclosed by Sambrook, *J., et al.*, In: *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989). Oligonucleotide molecules having a strand which encodes antisense RNA complementary to a TAL sequence can be

prepared using procedures which are well known to those of ordinary skill in the art. Details regarding such procedures are described in: Belagaje, R., *et al.*, *J. Biol. Chem. 254*:5765-5780 (1979); Maniatis, T., *et al.*, In: *MOLECULAR MECHANISMS IN THE CONTROL OF GENE EXPRESSION*, Nierlich, D.P., *et al.*, eds., Acad. Press, N.Y. (1976); Wu, R., *et al.*, *Prog. Nucl. Acid Res. Molec. Biol. 21*:101-141 (1978); Khorana, H.G., *Science 203*:614-625 (1979). Automated synthesizers may be used for DNA synthesis (such as are commercially available from Biosearch, Applied Biosystems, *etc.*). Techniques of nucleic acid hybridization are disclosed by Sambrook *et al.* (*supra*), and by Haymes, B.D., *et al.*, In: *NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH*, IRL Press, Washington, DC (1985).

Gene Delivery and Therapy Delivery Methods

A DNA molecule encoding TAL, such as disclosed by copending commonly assigned U.S. patent application 08/326,119, filed 19 October 1994, which is hereby incorporated by reference in its entirety) may be used for gene transfer to treat diseases or conditions wherein enhanced or suppressed TAL activity is desired, as is described above. In particular, this invention is useful for the treatment of a disease or condition in which it is desired to render cells more or less sensitive to apoptotic signals.

Two broad categories of gene transfer methods are utilized in the present invention: *in vivo* and *ex vivo* methods. In the latter, DNA transfer is performed *ex vivo* and the transfected cells are introduced into the subject animal.

Gene therapy involves introduction of a foreign gene into a cell and ultimately, into a live animal. Several general strategies for gene therapy have been studied and have been reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol. 12*:335-356 (1992); Anderson, W.F., *Science 256*:808-813 (1992); Miller, A.S., *Nature 357*:455-460

(1992); Crystal, R.G., *Amer. J. Med. 92(suppl 6A)*:44S-52S (1992); Zwiebel, J.A. *et al.*, *Ann. N.Y. Acad. Sci. 618*:394-404 (1991); McLachlin, J.R. *et al.*, *Prog. Nucl. Acid Res. Molec. Biol. 38*:91-135 (1990); Kohn, D.B. *et al.*, *Cancer Invest.* 7:179-192 (1989)).

For accomplishing the objectives of the present invention, gene therapy would be accomplished by direct transfer of the functionally active TAL DNA into mammalian somatic tissue or organ *in vivo*, and more preferably, into cells which are to be killed. DNA transfer can be achieved using a number of approaches described below. As is known in the art an optimal gene delivery system should bind the DNA and make it soluble, effectively transfer the DNA into the cell, protect it from nucleases, release the DNA for efficient activity, and be targetable to specific cells. The optimal system may differ according to the particular gene transfer application, *e.g.*, systemic versus local delivery, target cell type, *etc.*

In general, for transfer of DNA according to the present invention to achieve cell death by apoptosis, use of viral vectors is preferable to the use of plasmid DNA.

Examples of successful transfer of genes known in the art include: (a) direct injection of plasmid DNA into mouse muscle tissues leading to indefinite expression of marker genes (Wolff, J.A. *et al.*, *Science 247*:1465 (1990); Acsadi, G. *et al.*, *The New Biologist 3*:71 (1991)); (b) retroviral vectors effective for *in vivo* and *in situ* infection of blood vessel tissues; (c) portal vein injection and direct injection of retrovirus preparations into liver to effect gene transfer and expression *in vivo* (Horzaglou, M. *et al.*, *J. Biol. Chem. 265*:17285 (1990); Koleko, M. *et al.*, *Human Gene Therapy 2*:27 (1991); Ferry, N. *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8387 (1991)); (d) intratracheal infusion of recombinant adenovirus into lung tissues effective for *in vivo* transfer and prolonged expression of foreign genes

in lung respiratory epithelium (Rosenfeld, M.A. *et al.*, *Science 252*:431 (1991)); (e) Herpes simplex virus vectors for *in vivo* gene transfer into brain tissue (Ahmad, F. *et al.*, eds, *Miami Short Reports - Advances in Gene Technology: The Molecular Biology of Human Genetic Disease*, Vol 1, Boehringer Mannheim Biochemicals, USA, 1991).

Retroviral Vectors

Retroviral-mediated human gene therapy utilizes amphotrophic, replication-deficient retrovirus systems (Temin, H.M., *Human Gene Therapy 1*:111 (1990); Temin *et al.*, U.S. Patent 4,980,289; Temin *et al.*, U.S. Patent 4,650,764; Temin *et al.*, U.S. Patent No. 5,124,263; Wills, J.W. U.S. Patent 5,175,099; Miller, A.D., U.S. Patent No. 4,861,719; Miller, A.D., *Curr. Top. Microbiol. Immunol. 158*:1-24 (1989)). Such vectors have been used to introduce functional DNA into human cells or tissues, for example, the adenosine deaminase gene into lymphocytes, the NPT-II gene and the TNF gene into tumor infiltrating lymphocytes. Retrovirus-mediated gene delivery generally requires target cell proliferation for gene transfer (Miller, D.G. *et al.*, *Mol. Cell. Biol. 10*:4239 (1990)). This condition is met by the preferred target cells for the present invention, *i.e.*, growing tumor cells or activated lymphocytes in the case of autoimmunity. Gene therapy of cystic fibrosis using transfection by plasmids using any of a number of methods and by retroviral vectors has been described by Collins *et al.*, U.S. Patent 5,240,846

For use in the present methods, DNA encoding TAL (or TAL antisense DNA) is packaged into retrovirus vectors using one of several known packaging cell lines that produce replication-defective retroviruses (see, for example, Cone, R.D. *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6349-6353 (1984); Mann, R.F. *et al.*, Cell 33:153-159 (1983); Miller, A.D. *et al.*, *Molec. Cell. Biol.* 5:431-437 (1985); Sorge, J., *et al.*, *Molec. Cell. Biol.* 4:1730-1737

(1984); Hock, R.A. *et al.*, *Nature 320*:257 (1986); Miller, A.D. *et al.*, *Molec. Cell. Biol. 6*:2895-2902 (1986)). Newer packaging cell lines which are efficient and safe for gene transfer have been described more recently (Bank *et al.*, U.S. 5,278,056).

The gene therapy approach can be utilized in a site specific manner to deliver a retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel, E.G. *et al.*, *Science 244*:1342 (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the gene to a blood vessel wall, or into the blood circulation of a tumor.

Other Viral Vectors

Other virus vectors may also be used, in particular for human gene therapy, including recombinant adenovirus vectors (Horowitz, M.S., In: *VIROLOGY*, Fields, B.N. *et al.*, eds, Raven Press, New York, 1990, p. 1679; Berkner, K.L., *Biotechniques 6*:616-629 (1988), Strauss, S.E., In: *THE ADENOVIRUSES*, Ginsberg, H.S., ed., Plenum Press, New York, 1984, chapter 11) or adeno-associated virus (AAV) (Ohi, S. *et al.*, Gene *89*:279-282 (1990); Dixit, M. *et al.*, Gene 104:253-257 (1991); Samulski, R.J. *et al.*, *EMBO J. 10*:3941 (1991)). Herpes simplex virus (HSV) is well-adapted for neuron-specific delivery (Geller, A.I. *et al.*, *Science 241*:1667-1669 (1988)). Advantages of adenovirus vectors for human gene therapy include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organism.

Another vector which can express the TAL DNA molecule of the present invention, and is useful in gene therapy, particularly in humans, is vaccinia virus, which can be rendered non-replicating (U.S. Patents 5,225,336;

5,204,243; 5,155,020; 4,769,330). Descriptions of recombinant vaccinia viruses containing heterologous DNA and their uses in immunization and gene therapy are reviewed in: Moss, B., *Curr. Opin. Genet. Dev.* (1993) *3*:86-90; Moss, B. *Biotechnology* (1992) *20*: 345-362; Moss, B., *Curr Top Microbiol Immunol* (1992) *158*:25-38; Moss, B., *Science* (1991) *252*:1662-1667; Piccini, A *et al.*, *Adv. Virus Res.* (1988) 34:43-64; Moss, B. *et al.*, *Gene Amplif Anal* (1983) 3:201-213.

A nontoxic and efficient method has recently been reported based on the Sendai virus, also known as hemagglutinating virus of Japan (HVJ). HVJ-liposome-mediated gene transfer is performed in Morishita R *et al.*, *Hypertension* (1993) *21*:894. This method was used to transfect vascular smooth muscle cells and COS cells with human angiotensin converting enzyme (ACE) more effectively that by lipofection. Tomita N *et al.*, *Biochem Biophys Res Commun* (1992) *186*:129-134 developed a method in which plasmid DNA and high mobility group 1 protein (a nuclear protein) are co-encapsulated in liposomes and co-introduced into target cells by HVJ-mediated membrane fusion. This is a general method in which foreign genes and nuclear proteins are encapsulated into the same liposomes, which are then treated with inactivated HVJ. In this method, HVJ enables foreign genes to be introduced directly into the cytoplasm by membrane fusion and the nuclear proteins transport the foreign genes rapidly into the nuclei. In this study, a reporter gene, was introduced into the kidney of intact rats through a cannula in the renal artery. Tomita N *et al.*. *Cancer Detect Prev* (1994) 18:485-491 shows the successful introduction and expression of a human insulin gene in the mouse, with presence of human insulin in the mouse plasma and its reduction of plasma glucose levels. The human renin gene was similarly introduced into adult rat liver resulting in significant elevation of blood pressure for 6 days

compared with controls (Tomita, N. *et al.*, *Circ Res* (1993) 73:898-905).

Artificial Viral Envelopes

Based on the concept of viral mimicry, artificial viral envelopes (AVE) are designed based on the structure and composition of a viral membrane, such as HIV-1 or RSV and used to deliver genes into cells *in vitro* and *in vivo*. See, for example, U.S. 5,252,348, Schreier H. *et al.*, *J Mol. Recognit.*, 1995, *8*:59-62; Schreier H *et al.*, *J. Biol. Chem.*, 1994, 269:9090-9098; Schreier, H., *Pharm. Acta Helv.* 1994, 68:145-159; Chander, R. *et al. Life Sci.*, 1992, 50:481-489. The envelope is preferably produced in a two-step dialysis procedure where the naked envelope is formed initially, followed by unidirectional insertion of the viral surface glycoprotein of interest. This process and the physical characteristics of the resulting AVE are described in detail by Chander *et al.*, *(supra)*. Examples of AVE systems are (a) an AVE containing the HIV-1 surface glycoprotein gp160 (Chander *et al.*, *supra*; Schreier *et al.*, 1995, *supra*) or glycosyl phosphatidylinositol (GPI)-linked gp120 (Schreier *et al.*, 1994, *supra*), respectively, and (b) an AVE containing the respiratory syncytial virus (RSV) attachment (G) and fusion (F) glycoproteins (Stecenko, A.A. *et al.*, *Pharm. Pharmacol. Lett. 1*:127-129 (1992)). The viral functions of surface insertion and conformational integrity of the gp160 have been confirmed by sandwich immunolabelling with anti-gp160 mAb and colloidal gold carrying mouse anti-IgG. Selective delivery of contents to $CD4^+$-cells has been demonstrated. AVEs may be tested by loading with FITC-dextran and incubating with a population of target cells. For example, using the HIV gp160-containing AVE cells that are predominantly $CD4^+$ (REX-1B), flow cytometric analysis demonstrated incorporation of the label (77% of REX-1B cells after 60 minutes) whereas, in a population largely lacking the viral receptor (KG-1; about 18% $CD4^+$), only 25% of cells take up label. Ricin A

at concentrations as low as 2 ng/ml arrested cell growth of CD4-positive MOLT-4 cells, whereas 8 ng/ml ricin A in solution had no effect on cell growth. The arrest of cell growth was reverted in the presence of excess anti-gp120 monoclonal antibody. Naked envelopes (without HIV-1 rgp160 inserted) show background levels of interaction with target cells, transferring material less efficiently and nonspecifically. For a detailed description, see also Schreier, 1995 (*supra*) and a report showing inhibitable binding of GPI-anchored gp120 AVE to CHO cells and 293 cells transfected with human CD4 or CD4-DAF, respectively (Schreier *et al.*, 1994, *supra*). Thus, vesicles are constructed which mimic the natural membranes of enveloped viruses in their ability to bind to and deliver materials to cells bearing corresponding surface receptors.

AVEs are used to deliver genes both by intravenous injection and by instillation in the lungs. For example, AVEs are manufactured to mimic RSV, exhibiting the RSV F surface glycoprotein which provides selective entry into epithelial cells. F-AVE are loaded with a plasmid coding for the TAL (or a reporter gene such as CAT not present in mammalian tissue). Recipient animals, preferably humans, have an effective dose of the gene instilled into their lungs via a syringe connected to a thin endotracheal tube or, more preferably by inhalation. When carried out in rats using CAT as a reporter gene, animals sacrificed 48 hr after instillation, lungs showed significant activity of CAT above background. Though there was practically no expression in the liver, transfection and expression occurred in the kidney, most likely due to lymphatic delivery of the gene product to the blood and then to the kidney. In a similar study, rats were injected intravenously with AVEs carrying on their surface a specific lung targeting molecule (which has a high binding affinity to the surface of lung endothelial cells) and carrying a payload of a plasmid

encoding (heat-resistant) placental alkaline phosphatase (PAP). Anesthetized rats were injected with 600 μl L-AVE containing 100 μg of the gene product via a tail vein. Organs were analyzed after 60 hours for the presence of the PAP gene using a histochemical method which resulted in red precipitate in transfected cells. Extensive staining of lung tissue occurred, indicating efficient delivery to the targeted endothelial cells. Thus, both target selectivity as well as high efficiency of gene delivery have been unequivocally demonstrated *in vivo* using AVE.

The AVE system described herein is physically and chemically essentially identical to the natural virus yet is entirely artificial, as it is constructed from phospholipids, cholesterol, and recombinant viral surface glycoproteins. Hence, there is no carry-over of viral genetic information and no danger of inadvertent viral infection. Construction of the AVEs in two independent steps allows for bulk production of the plain lipid envelopes which, in a separate second step, can then be marked with the desired viral glycoprotein, also allowing for the preparation of protein cocktail formulations if desired.

Bacterial Delivery

Recently attenuated Shigella bacteria were described as a DNA delivery system (Sizemore, D.R. *et al.*, *Science* 270:299-302 (1995)). This approach exploits the ability of *Shigellae* to enter epithelial cells and escape the phagocytic vacuole as a method for delivering the TAL DNA construct into the cytoplasm of the target cell. Invasion with as few as one to five bacteria can result in expression of the foreign plasmid DNA delivered by these bacteria. As little as 4-20 x 10^9 μg DNA by Shigella was shown to be sufficient for expression of a transfected marker. Such Shigella-mediated delivery of plasmid DNA can be achieved in various cell types which can be infected by Shigella. Plasmid DNA has been

successfully delivered to animals *in vivo*, including keratoconjunctival delivery in guinea pig and intranasal delivery in mice. It is important that the delivery be done using highly attenuated bacteria for reasons of safety in human subjects. Importantly, this approach is not restricted to Shigella. Shigella invasion genes can be transferred to other bacterial genera such as *E. coli*. Other bacteria such as Listeria can invade cells and break out of phagocytic vacuoles and enter the cytoplasm. An important advantage of this approach for gene therapy is the ease and acceptability of oral and other forms of mucosal delivery.

Non-viral and Liposome Mediated Delivery

In addition to virus-mediated or bacterially-mediated gene transfer *in vivo*, physical means well-known in the art can be used for direct gene transfer, including administration of plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer (Yang, N.-S., *et al.*, *Proc. Natl. Acad. Sci. USA* *87*:9568 (1990); Williams, R.S. *et al.*, *Proc. Natl. Acad. Sci. USA* *88*:2726 (1991); Zelenin, A.V. *et al.*, *FEBS Lett.* *280*:94 (1991); Zelenin, A.V. *et al.*, *FEBS Lett.* *244*:65 (1989); Johnston, S.A. *et al.*, *In Vitro Cell. Dev. Biol.* *27*:11 (1991)). Furthermore, electroporation, a well-known means to transfer genes into cells *in vitro*, can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, A.V. *et al.*, *Biochim. Biophys. Acta* *1088*:131 ((1991)). In order to overcome therapy-limiting toxicity, antigenicity and lack of expression of transgenes in nonreplicating cells, non-viral vectors may be used. Such a method of gene transfer is also known as carrier mediated gene transfer (Wu, C.H. *et al.*, *J. Biol. Chem.* *264*:16985 (1989); Wu, G.Y. *et al.*, *J. Biol. Chem.* *263*:14621 (1988); Soriano, P. *et al.*, *Proc. Natl. Acad. Sci. USA* *80*:7128 (1983); Wang, C-Y. *et al.*, *Proc. Natl. Acad. Sci. USA* *84*:7851 (1982); Wilson, J.M. *et al.*, *J. Biol. Chem.* *267*:963 (1992)).

Cationic Lipids

A preferred type of mediator of nonviral transfection *in vitro* and *in vivo* is cationic (ammonium derivatized) lipids. These positively charged lipids form complexes with negatively charged DNA, resulting in DNA charged neutralization and compaction. The complexes endocytosed upon association with the cell membrane, and the DNA somehow escapes the endosome, gaining access to the cytoplasm. Cationic lipid:DNA complexes appear highly stable under normal conditions. Studies of the cationic lipid DOTAP suggest the complex dissociates when the inner layer of the cell membrane is destabilized and anionic lipids from the inner layer displace DNA from the cationic lipid. Several cationic lipids are available commercially. Two of these, DMRI and DC-cholesterol, have been used in human clinical trials. First generation cationic lipids are less efficient than viral vectors. A few cationic lipid compounds (*Genetic Engineering News*, Nov. 15, 1995, pg. 1) are up to two logs more active in their ability to express a reporter gene (CAT) in mouse lung than the compounds used in earlier gene transfer trials for cystic fibrosis. Although such lipids are still relatively inefficient compared to adenovirus (two logs more DNA molecules are required to achieve an equivalent level of expression), DNA is easier to make than virus. The novel cationic lipid:DNA complexes are 500-fold more active than naked DNA. For delivery to lung, any inflammatory responses accompanying the liposome administration are reduced by changing the delivery mode to aerosol administration which distributes the dose more evenly.

One well-known method for effecting efficient DNA transfection is termed lipofection (Felgner, PL *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7417). Cationic liposomes have been successfully employed to express the CFTR protein in rats and to correct the chloride ion transport defect both in transgenic mice and

in human patients. In one embodiment, this method utilizes a synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). Small unilamellar liposomes containing DOTMA interact spontaneously with DNA to form lipid-DNA complexes with 100% entrapment of the DNA. DOTMA facilitated fusion of the complex with the membrane of cultured cells resulting in both uptake and expression of the DNA. The technique is considered simple, highly reproducible, and effective for both transient and stable expression of transfected DNA.

A method employing cationic liposomes useful for direct gene transfer in the therapy of cancer and other diseases is discussed by Farhood, H. *et al.*, *Ann N Y Acad Sci* (1994) 716:23-35. Cationic liposomes mediate efficient delivery of DNA and DNA/protein complex to mammalian cells *in vitro* and *in vivo*. Cationic cholesterol derivatives mixed with phosphatidylethanolamine and sonicated to form small unilamellar vesicles complex with DNA and mediate the entry into the cytosol from the endosome compartment. One of the liposome formulations, DC-Chol liposomes, has been used in a gene therapy clinical trial for melanoma. Such cationic liposomes were used for the delivery of trans-activating protein factors to regulate and control the expression of delivered transgenes in a protein dose-dependent manner. Human tumor cells selected for cis-platin resistance or isolated from patients who have failed cis-platin therapy are highly transfectable with cationic liposomes. Thus the present method of introducing TAL DNA into a tumor cells will permit serial therapy with cis-platin (or another conventional cancer chemotherapeutic agent) and the TAL gene therapy to eradicate malignancy.

The use of cationic liposomes may be combined with Adeno-associated (AAV)-based plasmids to introduce TAL DNA into cancer cells. This method has been used to

transfer the IL-2 gene in human prostate cancer (Vieweg, J *et al. Cancer Res* (1995) 55:2366-2372).

A method for transient expression of genes in normal colonic epithelium involves liposomal gene delivery by rectal catheter infusion. This approach has been used to express a reporter gene and the human *APC* tumor suppressor gene under control of a constitutive promoter in a rodent model (Westbrook CA *et al., Hum Mol Genet* (1994) 3:2005-2010). High efficiency transfection was achieved (close to 100% of epithelial cells expressing the introduced gene). Expression in this system was transient (consistent with the normal turnover time of gut epithelium). However, repeated treatments maintained expression. Importantly, for the purposes of inducing apoptosis as described herein, such transient overexpression of the TAL gene may be sufficient to achieve the desired cytotoxic effect.

In another embodiment of this invention, TAL DNA is introduced into cells by using targeted liposomes (Nicolau, C. *et al., Proc. Natl. Acad. Sci. USA 80*:1068 (1983); Soriano *et al., supra*) such as immunoliposomes, which can incorporate acylated monoclonal antibodies into the lipid bilayer (Wang *et al., supra*). Polyclonal antibodies and mAbs specific for various types of tumors, viral antigens or cell surface markers of various normal cell types are well-known in the art. Thus, the TAL DNA is specifically introduced into a selected type of target cell by means of an antibody selective for that cell type. Thus, for example, an antibody specific for a class or subclass of lymphocytes, or for a particular T cell receptor variable region, can be used to target the TAL DNA to a particular lymphocyte population in the treatment of autoimmunity. An antibody specific for a tumor associated antigen is used to target the therapeutic composition to cells of a tumor.

Cochleates

Proteoliposome delivery vesicles can be prepared by the protein-cochleate method. Self-assembling lipid-based complexes termed cochleate are used for *in vivo* DNA transfer (Gould-Fogerite, S. *et al.*, 1985, *Anal. Biochem. 148*:15-25; Mannino, R.J. *et al.*, 1988, *Biotechniques 6*:682-690; Papahadjopoulos, D. *et al.*, *Biochim. Biophys. Acta*, 1975, *394*:483-491). Cochleates are prepared by calcium-induced fusion of phosphatidyl serine-cholesterol liposomes (anionic) resulting in an insoluble jellyroll-like structure. The layers of the jellyroll are composed of alternating sheets of negatively charged phospholipid and calcium. Gould-Fogerite, S. *et al.*, *Gene*, 1989, *84*:429-438, discloses a system in which proteins mediating the entry of enveloped viruses into cells are integrated in the lipid bilayer, and materials are encapsulated at high efficiency within the aqueous interior of these vesicles. Proteoliposome-mediated delivery of proteins and drugs into entire populations of cells can be achieved in culture with this approach. Material can be delivered gradually by Sendai virus glycoprotein-containing proteoliposomes or synchronous delivery can be achieved by exposing cell-bound influenza glycoprotein vesicles briefly to low pH buffer. When DNA is encapsulated, chimeric proteoliposome gene-transfer vesicles (chimerasomes), which mediate high-efficiency gene transfer *in vitro* and *in vivo*, are produced. Stable expression of a bovine papilloma virus-based plasmid in tissue-cultured cells, at 100,000 times greater efficiency than calcium phosphate precipitation of DNA, has been achieved. Stable gene transfer and expression in mice has been obtained by subcutaneous injection of chimerasomes containing a plasmid. In contrast to liposomes, cochleates are solid, multilayered, lyophilizable precipitates containing no internal aqueous space. A cochleate may be considered a fusion intermediate frozen in time. Benefits of this structure

include its ability to provide protection from degradation for associated or encochleated molecules, the nontoxic, nonimmunogenic nature of its components, and its stability (it can be lyophilized). Animal studies show that oral delivery of DNA wrapped in cochleates can result in systemic responses. This was demonstrated using an 11 kb DNA plasmid encoding the *env*, *tat*, and *rev* genes of $HIV1_{IIIB}$ driven by a CMV promoter. Both oral and intramuscular administration of the DNA cochleates induced antigen-specific T helper cell responses and cytotoxic lymphocyte activity in mice.

Also useful are polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*) wherein the conjugate includes (a) a molecule recognizing the target tissue; and (b) a DNA binding compound, such as polylysine, to bind to the TAL DNA being transfected. This conjugate is then complexed with plasmid DNA using known methods for transfer.

To facilitate direct delivery of genes to muscle, polyvinyl-based polymer (PVP) formulations are used (*Genetic Engineering News*, Nov. 15, 1995, pg. 1). PVP is already used in FDA-approved injectable pharmaceutical formulations. The rationale is to enhance expression by protecting the DNA from degradation while retaining the flexibility to promote good dispersion throughout the muscle. Another desired property is interaction with DNA without condensing it into small particles, based on the expectation that condensation decreases the expression level compared to naked DNA. With a PVP formulation, a 5-to-10-fold increase was observed compared to naked DNA in the level of expression of a β-galactosidase reporter gene on direct administration to rat muscle, as well as improved DNA dispersion throughout the muscle. When a muscle-specific human growth hormone gene construct was administered to rats using the PVP formulation a significant biological effect over time was observed compared to controls.

For endothelial cell delivery, cationic lipid/colipid delivery systems may be used. For gene delivery to hepatocytes a key concern is to protect DNA in the circulation after systemic delivery before it reaches the liver. A glycopeptide delivery system has been used that incorporates a proprietary small condensing peptide (developed as an alternative to polylysine to condense and protect DNA and allow extravasation of the particles through the liver (*Genetic Engineering News*, Nov. 15, 1995, pg. 1)). The peptide is galactosylated to target the asialoglycoprotein receptor in order to promote high affinity and specificity of gene delivery to the hepatocytes. The prototype system incorporates an endosomal release agent (lytic peptide) and a hepatocyte-specific promoter. With this approach the efficiency of transfection *in vitro* approaches that achieved with adenovirus.

Dendrimers

Dendrimers, a macromolecular architecture, have become recognized as useful vectors for gene transfection (Haensler, J. *et al.*, *Bioconjug. Chem.* 4:372-379 (1993); Tomalia, D.A., *Sci. Amer.* 272:62-66 (1995); Bielinska, A. *et al.*, *J. Invest. Med.* 43 (Suppl. 2):330A (1995); Kukowska-Latallo, J. *et al.*, *FASEB J.* 9:A409 (1995); Bielinska, A. *et al.*, *FASEB J.* 9:A312 (1995)). Dendrimers are made up of precise three-dimensional branches called dendrons, with a structure that mimics the bifurcation of tree branches. For gene transfer research the focus has been on the star-burst PAMAM (polyamidoamine) family of dendrimers. These are spherical polymers (polycationic) built up like layers of an onion (each layer being referred to as a generation), with an outside surface of primary amines. The similar dimensions of dendrimers of seven to eight generations to histones (about 80-90Å) led to macromolecular structures for which size, shape, surface chemistry, flexibility and topology can be controlled. They are composed of

nanoscopic building blocks or modules, either passive or reactive, and they constitute a fourth new major class of macromolecular architecture (after linear, cross-linked and branched structures). Dendrimers are nonimmunogenic and appear to protect DNA against nucleases. To enhance transfection ability, an excess of dendrimer to DNA is preferred.

Pharmacological Regulation of Apoptosis by Modulation of Transaldolase and the Pentose Phosphate Pathway

In a preferred embodiment, small molecules are used to regulate the PPP, and through this regulation to modulate apoptosis. Such molecules may be substrates of TAL or of other enzymes in this pathway. For example, allopurinol, an analogue of hypoxanthine, or its metabolites, inhibits purine oxidase enzymes thereby inhibiting breakdown of purines. By acting upon the PPP, this results in buildup of ribose-5-phosphate and ultimately the upregulation of G6PD. This occurs via the action of TAL, which is involved in the conversion of ribose-5-phosphate to glucose-6-phosphate which is a substrate for G6PD. Hence, the PPP is subject to regulation and modulation by introduction of an agent which influences the TAL-catalyzed reaction.

Thus, allopurinol or substrates of TAL, in particular substrates which have been rendered stable by chemical modification to block their conversion by the enzyme, are the basis for novel methods for modulating the PPP, influencing TAL activity, and, through this, promoting or suppressing apoptosis. See Figure 11, which illustrates the PPP.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Materials and Methods

Reagents

Anti-Fas monoclonal antibody CH-11 was obtained from MBL (Watertown, MA). Human recombinant TNF-α was obtained from Fisher (Pittsburgh, PA). Hydrogen peroxide (H_2O_2), sodium nitroprusside (SNP), N-acetyl cysteine (NAC), buthionine sulfoximine (BSO), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and 3,3,5,5-tetramethyl-1-pyrroline-1-oxide (TMPO), desferrioxamine (DEF), nordihydroguaiaretic acid (NDGA), Amytal, and all reagents for the enzyme, NADH, NADPH and GSH assays were from Sigma (St. Louis, MO). Anti-human actin monoclonal antibody was from Boehringer-Mannheim (Indianapolis, IN). 5,6-carboxy-2,7-dichlorofluorescein-diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR) were obtained from Molecular Probes (Eugene, OR).

Construction and transfection of eukaryotic expression vectors

TAL-H cDNA clone 4/2-4/1 (18) was inserted into the HpaI site of the metallothionein promoter-driven pMAXRHneo-1 vector (19) following removal of the coding sequence of the p40/tax protein of HTLV-I by cleavage with HpaI. Clones containing the TAL-H cDNA in the sense (pL26-3) and antisense orientation (pL18-3) were selected. 20 μg of plasmid DNA linearized with KpnI were used to stably transfect Jurkat cells by electroporation as described (19). Transfected cells were grown in the presence of 750 μg/ml G418 and cloned by limiting dilution. Levels of transaldolase expression were measured by enzyme assay and Western blot analyses in the absence and presence of 5 μM $CdCl_2$. TAL protein expression was assessed by Western blot analysis and compared to that of actin using an automated scanning densitometer (Bio-Rad Model GS-670). Transfection of the pMAXRHneo-1 vector alone had no effect on TAL activity. Due to a leakiness of the promoter, cell lines producing increased

and suppressed levels of TAL in the absence of $CdCl_2$ were obtained. While overexpression and suppression were doubled by incubating the cells with 5 μM $CdCl_2$, $CdCl_2$ was not utilized in the following experiments to preclude possible interference with apoptotic pathways (20).

Induction of apoptosis

Cells were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml gentamicin at 37°C in a humidified atmosphere with 5% CO_2. 24 h prior to assays, cells were fed with fresh medium and seeded at a density of 2 x 10^5 cells/ml. CD95/Fas/Apo-1-mediated cell death was induced with 50 or 100 ng/ml anti-Fas monoclonal antibody CH-11. TNF-mediated cell death was induced with 20 ng/ml or 100 ng/ml human recombinant TNF-α as earlier described (21-23). Apoptotic cell death was also induced by withdrawal of fetal calf serum or treatment with 50 μM or 100 μM H_2O_2, or 2.5, 5, and 10 mM sodium nitroprusside (SNP), used as a source of exogenous NO (4). During treatment with SNP cells were cultured in the dark (aluminum foil-covered plate). Apoptosis was monitored by observing cell shrinkage, nuclear fragmentation, and quantified by trypan blue exclusion (1). DNA fragmentation during apoptosis was monitored by agarose gel electrophoresis (24). Apoptosis was also measured by flow cytometry as previously described (25). Briefly, following induction of apoptosis, cells were washed in PBS and resuspended in PBS with 0.1% Triton X-100 and 50 μg/ml propidium iodide. The DNA content was analyzed by using a Beckon Dickinson FACStarplus flow-cytometer. Hypodiploid cells containing a lower amount of DNA and a side scatter higher than that of G0/G1 cells were considered to be apoptotic.

Transaldolase (TAL) activities

The reverse reaction catalyzed by TAL was tested in the presence of 3.2 mM D-fructose 6-phosphate, 0.2 mM erythrose 4-phosphate, 0.1 mM NADH, 10 μg

α-glycerophosphate dehydrogenase/triosephosphate isomerase at a 1:6 ratio in 40 mM triethanolamine pH 7.6, 5 mM EDTA at room temperature by continuous absorbance reading at 340 nm for 8 min (18). The forward reaction catalyzed by TAL was measured at room temperature in the presence of 50 mM TEA pH 7.4, 5mM $MgCl_2$, 3 mM ribose 5-phosphate, 0.9 mM xylulose 5-phosphate, 0.5 mM NADP, 0.2 mM thiamine pyrophosphate, 0.2 U/ml transketolase, 0.4 U/ml phosphoglucose isomerase, and 0.3 U/ml G6PD, following a 10 min lag period, by continuous absorbance reading at 340 nm for 8 min (26). The enzyme assays were conducted in the activity range of 0.001-0.01 U/ml. Unless indicated otherwise, TAL activity refers to enzyme activity measurements conducted in the reverse reaction.

Enzymatic Activities of Transketolase, glucose 6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (6PGD)

Transketolase (TK) activity was measured in 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.06 mM thiamine pyrophosphate, 0.1 mM NADH, 10 μg α-glycerophosphate dehydrogenase/triosephosphate isomerase at a 1:6 ratio, 5 mM ribose 5-phosphate, and 1.25 mM xylulose 5-phosphate (13). G6PD was measured in the presence of 120 mM Tris pH 8.4, 10 mM $MgCl_2$, 2 mM glucose 6-phosphate, 0.9 mM NADP, and 0.1 U/ml 6PGD (27). 6PGD activity was determined in 120 mM Tris pH 7.7, 10 mM $MgCl_2$, 0.9 mM NADP, 2 mM 6-phosphogluconate (27).

Glutathione, NADPH, and NADH levels

Total glutathione content was determined by the enzymatic recycling procedure essentially as described by Tietze (28). 10^6 cells were resuspended in 100 μl of 4.5% 5-sulfosalicylic acid. The acid-precipitated protein was pelleted by centrifugation at 4°C for 10 min at 2000 x g. The total protein content of each sample was determined using the Lowry assay (29). GSH content of the aliquot assayed was determined in comparison to reference curves generated with known amounts of GSH. For NADPH and NADH

assays 5 x 10^6 cells were washed in PBS and resuspended in 125 µl of H_2O and pyridine nucleotides were extracted by adding 62.5 µl of freshly prepared 1 M KOH in ethanol as earlier described (30).

Infection with HIV-1

Jurkat-tat cells was transfected with HIV-1 DNA clone 4803 (54) by electroporation at 600µF/250V/72Ω. Infectious stock of the strain HIV-14803 was harvested from 24 h supernatants of freshly re-infected Jurkat-tat cells and infectious titer was determined by an *in situ* infectivity (MAGI) assay (Nagy, K. *et al.*, (1994) *J. Virol. 68*:757-765). Supernatants with titers of 2.1 x 10^5 infectious units (IU)/ml were filtered through 0.45µ filter and aliquots were stored at -70°C. L26-3/4, L18-3/1 and control Jurkat cells were incubated for 4 hr with HIV-1 in the presence of 10 µg/ml Polybrene (Sigma). Infections were standardized by incubating with cell-free virus supernatants containing 200 ng of p24 core protein per 5 x 10^6 cells as earlier described (16). Normal human PBL purified on Ficoll-Hypaque gradient were prestimulated with 1 µg/ml phytohemagglutinin (PHA, Sigma) and 50 U/ml human recombinant interleukin-2 in RPMI 1640 medium containing 20% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. After three days, PBL were incubated for 4 hr with HIV-1 in the presence of 10 µg/ml Polybrene as described for Jurkat-tat cells. After virus infection, cells were washed in PBS and resuspended in 10 ml of fresh RPMI 1640 medium containing 20% fetal calf serum, 50 U/ml IL-2, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Non-infected control PBL were cultured under identical conditions. Transmission of HIV-1 was monitored by production of gagp24. Recombinant HIV-1 gag24 protein was utilized as a control antigen. As positive control sera, HIV-1 gag p24 specific polyclonal sheep antibody and monoclonal antibodies to p24 were utilized. Viral reagents were

obtained from the National Institutes of Health AIDS Research and Reference Program.

Western blot analysis

Protein lysates were prepared from cell lines and quantified by the Lowry method. 40 μg of protein lysate in 10 μl per well was separated by SDS-PAGE and electroblotted to nitrocellulose. Nitrocellulose strips were incubated in 100 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20, and 5% skim milk with TAL-H-specific rabbit Ab 169 (18) or actin-specific murine monoclonal antibody C4 at a 1000-fold dilution at room temperature overnight. For detection of rabbit antibodies, after washing, the strips were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN). For detection of murine antibodies, after washing, the strips were incubated with biotinylated goat anti-mouse serum and, subsequently with horseradish peroxidase-conjugated avidin (Jackson Laboratories, West Grove, PA). In between the incubations the strips were vigorously washed in 0.1% Tween-20, 100 mM Tris pH 7.5, and 0.9% NaCl. The blots were developed with a substrate comprised of 1 mg/ml 4-chloronaphthol and 0.003% hydrogen peroxide.

Flow cytometric analysis of the rates of intracellular oxidation

The production of ROIs was estimated fluorometrically using 5,6-carboxy-2,7-dichlorofluorescein-diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR) as earlier described (31,32). Following apoptosis assay, cells were washed three times in 5 mM Hepes-buffered saline (HBS), pH 7.4, incubated in HBS with 0.1 μM DCFH-DA or DHR for 2 min, and samples were analyzed using a Beckon Dickinson FACStar Plus flow cytometer equipped with an argon ion laser delivering 200 mW of power at 488 nm. Fluorescence emission from 5,6-carboxy-2,7-dichlorofluorescein (DCF; green) or DHR (green) was detected at a wavelength of 530 ± 30 nm. Dead

cells and debris were excluded from the analysis by electronic gating of forward and side scatter measurements.

Statistical Analysis

Alterations in cell survival, NADPH, NADH, and GSH levels, and activity of PPP enzymes were analyzed by Student's t-test. Changes were considered significant at $p < 0.05$.

EXAMPLE II

Regulation of the PPP and GSH levels by TAL

To assess how TAL expression influences biochemical processes regulating apoptosis, cell lines producing increased and suppressed levels of TAL were generated. Jurkat human T cells were stably transfected with eukaryotic expression vectors containing full length transaldolase cDNA in the sense (pL26-3) or antisense orientation (pL18-3). Western blot analysis revealed several cell lines carrying pL26-3 and pL18-3 which had either increased or decreased levels of TAL expression, respectively. Using actin as a baseline, TAL expression was increased by 160 ± 9% (2.6-fold) in L26-3/4 cells (Figure 1) and 47 ± 8% in L26-3/2D1 cells, whereas, TAL activity was suppressed by 25 ± 4% in L18-3/1 cells (Figure 1) and 55 ± 6% in L18-3/1D9 cells in comparison to control Jurkat cells. Transfection with the plasmid vector lacking TAL-H cDNA had no effect on enzymatic activities in pXH1C6 and pXH2D2 cells (Table 2).

In correlation with the levels of TAL expression, enzymatic activities were increased in L26-3/4 cells and L26-3/2D1 cells, whereas, TAL activity was decreased in L18-3/1 cells and L18-3/1D9 cells (Table 2).

Maximal velocity of TAL activity in the forward reaction was increased by 127% (2.3-fold) in L26-3/4 cells and by 24.9% (1.25-fold) in L26-3/2D1 cells. TAL activity in the reverse reaction was augmented by 210%

(3.1-fold) in L26-3/4 cells and by 24.9% (1.25-fold) in L26-3/2D1 cells.

The effects of TAL expression on activities of other key PPP enzymes and intracellular levels of NADPH, NADH, and GSH were evaluated. Overexpression and increased activity of TAL had no significant effect on TK activities in L26-3/4 and L26-3/2D1 cells. Suppression of TAL was associated with a decrease of TK activity in L18-3/1 cells while TK activity remained unchanged in L18-3/1D9 cells. Since the effect of TAL suppression was greater in L18-3/1D9 than in L18-3/1 cells, reduction of TK in L18-3/1 cells may not be related to diminished TAL activity.

Activities of enzymes of the oxidative branch of the PPP, G6PD and 6PGD, which are directly responsible for NADPH production, were inversely correlated with TAL expression (Table 2). Accordingly, NADPH levels were decreased in L26-3/4 and L26-3/2D1 cells in comparison to control Jurkat cells. GSH levels were depleted in these cell lines. NADH levels were also diminished in L26-3/4 and L26-3/2D1 cells. In conclusion, increased TAL expression occurred with coordinated changes which included (a) down-regulation of G6PD and 6PGD activities and (b) a decrease of NADPH and GSH synthesis in both L26-3/4 and L26-3/2D1 cells. Along the same line, decreased TAL expression occurred with upregulation of G6PD and 6PGD activities and an increase of GSH levels in L18-3/4 and L18-3/1D9 cells. However, NADPH and NADH levels were unchanged in these cell lines.

EXAMPLE III

Sensitivity to apoptotic signals is influenced by levels of TAL expression

Stably transfected cell lines with increased and depressed TAL activities have been maintained in culture for over two years showing viability similar to that of control Jurkat cells (>99%). However, levels of TAL

expression had a dramatic influence on susceptibility to apoptotic cell death induced by (1) H_2O_2, (2) NO, (3) TNFα, (4) anti-Fas monoclonal antibody, or (5) withdrawal of fetal calf serum from the culture medium (Figure 2). Apoptosis was monitored by DNA ladder formation (Figure 3) and flow cytometry as shown for Fas simulation (Figure 4). Cell death was particularly accelerated in TAL-overproducing cells in comparison to control Jurkat cells. Suppressed TAL expression inhibited cell death produced by all stimuli tested. Cell survival inversely correlated with TAL expression levels (Table 2).

HIV-Induced Apoptosis is Regulated by TAL

The level of TAL expression influenced the rate of HIV-induced cell death in Jurkat cells infected with HIV-1. While increased expression of TAL through overexpression of TAL-encoding DNA accelerated cell death, decreased expression of TAL by expression of TAL antisense DNA inhibited cell death (Figure 9).

Cell death programs induced by Fas or TNF stimulation or by HIV-1 infection caused alterations of PPP enzyme activities leading to depleted intracellular GSH in a pattern reminiscent of the changes produced by TAL overexpression.

EXAMPLE IV

Fas-mediated Apoptosis is Associated with Production of ROI

The effect of TAL expression on susceptibility to apoptosis through regulating the PPP and GSH production suggested the involvement of ROIs in each of the pathways tested. While production of ROIs has been associated with apoptosis induced by H_2O_2, NO, TNF, and serum deprivation, involvement of ROIs in Fas-dependent signaling has been controversial.

To assess changes in intracellular ROIs, oxidation-sensitive fluorescent probes DCFH-DA and DHR (32,33) were used. DCFH-DA is nonfluorescent, readily accumulates

within cells and, following deacetylation to DCFH, is oxidized to the fluorescent compound DCF. Similarly, DHR is nonfluorescent, uncharged, and readily taken up by cells, whereas R123, the product of DHR oxidation, is fluorescent, positively charged and trapped within cells. The rates of increase in fluorescence of cells treated with 100 μM H_2O_2 and 50 ng/ml anti-Fas mAb were evaluated. As shown in Figure 5, relative to H_2O_2 a smaller but consistent increase in ROI was detected in Fas-stimulated cells as compared with untreated cells. In agreement with earlier data (33), DHR was a significantly more sensitive detector of increases in ROI levels than DCFH. Production of ROI correlated with the rate of cell death ($p < 0.01$, Figure 6).

EXAMPLE V

Effect of Antioxidants on TAL and G6PD Activities, GSH Levels and Fas-induced Cell Death

The involvement of ROI in Fas-dependent signaling was suggested by the above observation of (i) an increased sensitivity to Fas-induced death of cells with increased TAL expression and decreased GSH content and (ii) the production of ROI during Fas-mediated apoptosis. An examination of whether changes in GSH levels influence Fas-induced PCD was therefore done. Under the conditions utilized, none of the agents had significant effect on binding of anti-Fas antibody to its receptor (measured using flow cytometry). Intracellular GSH levels were raised by as much as two-fold using NAC, a precursor of glutathione (34), or suppressed to less than 15% of baseline by buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl-cysteine synthetase (34) (Figure 7). Neither NAC nor BSO influenced TAL activity. In contrast, G6PD activity was increased by both NAC ($p < 0.01$) and BSO ($p < 0.05$) after 24 hr incubation. In accordance with earlier observations (35), cell viability was not affected up to 4 days in culture by either NAC

(up to 3 mM), BSO (up to 1 mM), or any of the other antioxidants tested. However, Fas-mediated cell death was markedly influenced by NAC and BSO (Figure 8). Prior to stimulation with anti-Fas antibody, Jurkat cells were pretreated with NAC or BSO for 24 h. 1 mM BSO substantially accelerated apoptosis, while 3 mM NAC inhibited Fas-mediated cell death ($p < 0.01$; Figure 8). The antioxidants amytal, desferrioxamine, and nordihydroguaiaretic acid also inhibited Fas-induced apoptosis (Figure 8).

DMPO and TMPO are nitrones which react with ROIs to form more stable nitroxide radical products (36) and have been shown to protect thymocytes against apoptosis (9). While DMPO or TMPO had no significant effect on GSH levels, they also inhibited Fas-induced apoptosis ($p < 0.01$; Figure 6B(8)).

EXAMPLE VI

Phosphorylation Stimulates TAL Activity

Radioimmunoprecipitation studies revealed that TAL-H is phosphorylated in resting human Jurkat and CEM T cells. Furthermore, phosphorylation of TAL-H was increased at least 3-fold after incubating the cells 5 min. with phorbol myristate acetate (PMA), a specific stimulator of protein kinase C or with a calcium ionophore which stimulates calmodulin-dependent kinase II. (PMA and the calcium ionophore were obtained from GIBCO-BRL). Increased phosphorylation was accompanied by an up to three-fold stimulation of TAL enzymatic activity in a whole cell lysate of Jurkat cells. Similarly, enzymatic activity of recombinant TAL was increased 2.5-fold by phosphorylation with protein kinase C (see Figure 10). The presence of an inhibitor of PKC during this pretreatment fully reversed the effect, indicating that phosphorylation was the basis of the effect.

PMA and calcium ionophore are known to be potent inducers of apoptosis (*Immunol. Rev.* 1994, *142*:301-320),

indicating that phosphorylation of TAL-H can serve as a key signaling mechanism of certain forms of PCD.

EXAMPLE VII

The nucleotide sequence of the human transaldolase mRNA is provided herein as SEQ ID NO:1. This sequence has been assigned Genbank Accession No. L19437. Antisense oligonucleotides are designed based upon this sequence.

Discussion of Results in Examples II-V

Overexpression of TAL in Jurkat human T cells resulted in down-regulation of G6PD and 6PGD activities and a decrease of NADPH and GSH levels. NADH levels were also reduced in TAL-overproducing cells which was consistent with the tendency to maintain NADPH at the expense of NADH by transhydrogenases (37). Alternatively, decreased TAL expression led to upregulation of G6PD and 6PGD activities and increased GSH levels.

Sensitivity to apoptosis was effectively controlled by regulating the activity of TAL, a pivotal enzyme of the PPP. Overexpression of TAL increased sensitivity, while suppression of TAL decreased sensitivity to six different apoptotic signals, indicating that TAL expression levels profoundly influence susceptibility to PCD.

The mechanism of this regulatory function may be explained by a considerable difference in forward TAL catalytic activity which favors production of glucose 6-phosphate, compared to reverse TAL activity which depletes glucose 6-phosphate (11). Studies in yeast showed that the maximal velocity of TAL in the forward direction was only about one third the velocity of TAL in the reverse direction (38). Reversibility of the TAL reaction was proposed as a possible control mechanism for the entire pathway in yeast (26).

In L26-3/4 and L26/2D1 cells overexpressing TAL the nonoxidative branch was pushed in the reverse direction resulting in the depletion of glucose 6-phosphate. This effect may be directly responsible for diminished G6PD activities and GSH levels and increased sensitivity to apoptotic signals.

The involvement of ROIs in each of the apoptosis signaling pathways examined here was suggested by the finding that TAL expression, via regulation of the PPP and of GSH production, modulated susceptibility to apoptosis. Apoptosis triggered by serum withdrawal (39), and NO has been associated with production of ROIs (4). Because GSH is the most abundant intracellular thiol compound which neutralizes ROI, a direct correlation of GSH levels with resistance to apoptosis induced by either H_2O_2, NO, or serum withdrawal was obtained. In contrast, an involvement of ROIs has not been clearly defined in Fas and TNF-induced apoptotic pathways which are signaled through specific cell surface receptors. The Fas/Apo-1 antigen and the TNFα receptor are members of the TNF/nerve growth factor receptor superfamily. All these receptors contain canonical cysteine-rich extracellular domains; Fas and the type I TNF receptor, mainly responsible for mediating cytolytic activity of TNF, also have a common 70 amino acid intracellular sequence which may play a role in triggering common cytoplasmic death signals. Indeed, TNF-mediated apoptosis is known to involve oxidative stress based on (a) the induction of ROI in response to TNF (40,41) and (b) the inhibition of TNF-induced killing by free radical scavengers (40-42). TNF- but not Fas-mediated cell death could be inhibited by ROI-scavenging compounds (42). No requirement of ROIs in either TNF- or Fas-mediated apoptosis was suggested by Hug *et al.* (43). In contrast, the effect of deletion of the cytoplasmic death domains shared by Fas and type I TNF receptor and involvement of common signal transducers

suggests that both receptors may mediate cell death via similar mechanism (44).

Since apoptosis induced by stimulation of the Fas antigen or by infection with HIV-1 resulted in similarly altered PPP enzyme activities and depleted intracellular GSH, the effect of Fas receptor blocking on HIV-induced cell death was analyzed. The inhibitory IgG mAb, ZB4 (which completely abrogated apoptosis induced by the anti-Fas mAb CH-11) failed to block HIV-induced cell death. The specificity of this response was supported by the finding that pretreatment with a mAb to the human TNF receptor had no effect on HIV-induced cell death. Hence, HIV-induced apoptosis does not appear to proceed through the Fas antigen or the TNF receptor. However, ZB4, which did not affect viability of control Jurkat or Jurkat-tat cells, did accelerate HIV-induced apoptosis, consistent with the observation that binding of HIV gp120 to CD4 (or other interactions between viral proteins and the T cell surface) may initiate a cell death program (2) and sensitize cells to apoptosis induced by a suboptimal or partial apoptotic stimulus such as an inherently non-cytolytic anti-Fas antibody like ZB4.

Indeed, the results presented herein indicated that the rate of both TNF and Fas-mediated cell death programs correlated with intracellular GSH levels as regulated by TAL expression. Increased production of ROI by Fas-mediated signaling was demonstrated using oxidation-sensitive fluorescent probes, DCFH-DA and DHR. DHR was found to be a much more sensitive probe of ROI than DCFH-DA (Figures 5 and 6) since the product of DHR oxidation, R123, is effectively trapped within the cell (33). In correlation with the rate of Fas-induced cell death, production of ROIs was accelerated in L26-3/4 and L26-3/2D1 cells while intracellular ROI levels were not significantly increased in L18-3/1 and L18-1/1D9 cells. Moreover, several different pretreatment regimens, NAC which increases GSH levels, DMPO and TMPO, two free

radical spin traps which form relatively stable nitroxide radical products with ROIs (40), and various antioxidants (DEF, NDGA, and Amytal) all protected against cell death.

The present observation of greatly accelerated Fas-induced killing following depletion of GSH by BSO provides additional evidence for the involvement of ROI in Fas-dependent signaling. Thus, cells with higher GSH levels were less sensitive, whereas cells with diminished GSH levels were more sensitive to Fas-mediated apoptosis. The Fas signaling pathway is concluded to be also associated with the formation of ROIs.

The present results support the notion that the PPP is an important biochemical mechanism regulating sensitivity to cell death programs dependent on formation of ROIs. This is consistent with an essential role of the PPP in the generation of NADPH for the synthesis of GSH which, in turn, protects cellular integrity from oxygen radicals. Levels of TAL expression may have a dominant role in regulating the balance between the two branches of PPP and the ultimate output of GSH. It is concluded that TAL, and possibly other PPP enzymes, serves as a critical determinant of tissue and cell type-specific sensitivity to apoptotic signals.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

Table 1

DISEASES ASSOCIATED WITH INDUCTION OR INHIBITION OF APOPTOTIC CELL DEATH

INHIBITION OF APOPTOSIS

1. Cancer
   - Carcinomas with p53 mutations
     - Follicular lymphomas
   - Hormone-dependent tumors
   - Breast cancer
   - Prostate cancer
   - Ovarian cancer
2. Autoimmune disorders
3. Viral infections
   - Herpesviruses
   - Poxviruses
   - Adenoviruses

INCREASED APOPTOSIS

1. AIDS
2. Demyelinating Diseases
   - Multiple sclerosis (MS)
3. Neurodegenerative disorders
   - Alzheimers disease
   - Parkinsons disease
   - Amyotrophic lateral sclerosis
   - Retinitis pigmentosa
   - Cerebellar degeneration
4. Myelodysplastic syndromes
   - Aplastic anemia
5. Ischemic injury
   - Myocardial infarction
   - Stroke
6. Toxin-induced liver diseases
   - Alcoholism

TABLE 2

Measurement of the activity of TAL in the forward (FOR) and reverse (REV) directions, TK, G6PD, and 6PGD (mU/mg protein), and of the levels of NADH (pmol/µg protein), NADPH (pmol/µg protein) and GSH (pmol/µg protein) in Jurkat cells stably transfected with TAL-H expression vectors.

Cell	TAL-FOR	TAL-REV	(FOR-REV)[1]	TK	G6PD	6PGD	NADH	NADPH	GSH	% viability
Jurkat	22.5 ± 0.9	20.8 ± 1.0	1.7 ± 1.0	9.3 ± 0.5	39.6 ± 2.9	51.4 ± 2.6	0.33 ± 0.01	1.28 ± 0.1	81.2 ± 7.7	51.9 ± 4.3
pXH2D2	23.0 ± 1.3	20.1 ± 2.1	2.9 ± 2.0	9.0 ± 0.6	42.6 ± 7.1	44.3 ± 2.9	0.36 ± 0.06	1.12 ± 0.1	77.1 ± 9.1	44.1 ± 3.5
L26-3/4	51.8 ± 2.6‡	64.5 ± 6.1‡	-12.7 ± 2.8‡	9.4 ± 0.2	15.4 ±2.6‡	40.3 ±1.9 †	0.24 ± 0.01‡	0.77 ± 0.1‡	37.9 ± 6.8‡	20.1 ± 2.4‡
L26-3/ 2D1	28.1 ± 1.3†	32.4 ± 2.7†	-4.3 ± 1.8†	9.2 ± 0.3	35.6 ± 3.0	42.6 ± 3.2*	0.19 ± 0.03‡	0.58 ± 0.2‡	39.6 ± 9.1‡	32.0 ± 6.5‡
L18-3/1	17.7 ± 1.2†	14.4 ± 1.1‡	3.3 ± 1.3	4.9 ± 0.3‡	58.6 ± 3.6‡	66.8 ± 2.9‡	0.33 ± 0.02	0.99 ± 0.2	149.5 ± 22†	95.5 ± 3.4‡
L18-3/1D9	13.5 ± 0.9‡	9.6 ± 2.4‡	3.9 ± 1.3	9.7 ± 0.3	48.6 ± 3.8*	58.0 ± 3.1*	0.28 ± 0.04	0.95 ± 0.2	123.2 ± 31†	82.0 ± 9.8‡

[1] (FOR–REV) represents the difference between the forward and reverse activities of TAL

L26-3/4 and L26-3/2D1 cells were transfected with the sense construct. L18-3/1 and L18-3/1D9 cells were transfected with the antisense construct. pXH2D2 cells were transfected with the expression vector without TAL-H cDNA. % survival was assessed by trypan blue exclusion after 24 h stimulation with 50 ng/ml anti-Fas monoclonal antibody. Data show mean ± SD of eight independent experiments. P values indicate significant differences in enzyme activities, NADH, NADPH and GSH levels in transfected cells as compared to those of Jurkat cells. *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$.

REFERENCES

1. Wyllie, A.H., et al. (1980) *Int Rev Cytol* **68**:251-306

2. Banda, N.K. et al. (1992) J Exp Med **176**:1099-1106

3. Thompson, C.B. (1995) *Science* **267**:1456-1462

4. Lipton, S.A., et al. (1993) *Nature* **364**:626-631

5. Kane, D.J., et al. (1993) *Science* **262**:1274-1277

6. Hockenberry, D.M., et al. (1993) *Cell* **75**:241-251

7. Buttke, T.M. and Sandstrom, P.A. (1994) *Immunol Today* **15**:7-10

8. Sarafian, T.A. and Bredesen, D.E. (1994) *Free Rad Res* **21**:1-8

9. Slater, A.F.G., et al. (1995) *Biochem J* **306**:771-778

10. Jacobson, M.D. and Raff, M.C. (1995) *Nature* **374**:814-816

11. Mayes, P. (1993) in *Harper's Biochemistry* (Murray, R., et al., eds) pp. 201-211, Appleton & Lange, Norwalk, CT

12. Wood, T. (1985) *The pentose phosphate pathway*, Academic Press, New York

13. Heinrich, P.C., et al. (1976) *Cancer Res* **36**:3189-3197

14. Novello, F. and McLean, P. (1968) *Biochem J* **107**:775-791

15. Baquer, N.Z., et al. (1977) *Developmental Medicine & Child Neurology* **19**:81-104

16. Banki, K., et al. (1994) *J Exp Med* **180**:1649-1663

17. Merrill, J.E., et al. (1993) *J Immunol* **151**:2132-2141

18. Banki, K., et al. (1994) *J Biol Chem* **269**:2847-2851

19. Banki, K., et al. (1994) *AIDS Res Hum Retrovirus* **10**:303-308

20. Halliwell, B. and Gutteridge, J.M. (1990) *Meth Enzymol* **186**:1-85

21. Yonehara, S., et al. (1989) *J Exp Med* **169**:1747-1756

22. Itoh, N., et al. (1991) *Cell* **66**:233-243

23. Trauth, B.C., et al. (1989) *Science* **245**:301-305

24. Perl, A., et al. (1989) *Nucl Acids Res* **17**:6841-6854

25. Nicoletti, I., et al. (1991) *J Immunol Methods* **139**:271-279

26. Venkataraman, R. and Racker, E. (1961) *J Biol Chem* **236**:1876-1882

27. Rudack, D., et al. (1971) *J Biol Chem* **246**:1249-1254

28. Tietze, F. (1969) *Anal Biochem* **27**:502-522

29. Lowry, O.H., et al. (1951) *J Biol Chem* **193**:265-275

30. Zerez, C.R., et al. (1987) *Anal Biochem* **164**:367-373

31. Burow, S. and Valet, G. (1987) *Eur J Cell Biol* **43**:128-133

32. Royall, J.A. and Ischiropoulos, H. (1993) *Arch Biochem Biophys* **302**:348-355

33. Packham, G., et al. (1996) *J Immunol* **156**:2792-2800

34. Meister, A. and Anderson, M.E. (1983) *Ann Rev Biochem* **52**:711-760

35. Hamilos, D.L. and Wedner, H.J. (1985) *J Immunol* **135**:2740-2747

36. Finkelstein, E., et al. (1980) *Arch Biochem Biophys* **200**:1-16

37. Rydstrom, J. (1977) *Biochim Biophys Acta* **463**:155-184

38. Wood, T. (1972) *FEBS Lett* **25**:153-155

39. Sandstrom, P.A. and Butke, T.M. (1993) *Proc Natl Acad Sci USA* **90**:4708-4712

40. Meier, B., et al. (1989) *Biochem J* **263**:539-545

41. Hennet, T., et al. (1993) *Biochem J* **289**:587-592

42. Schulze-Osthoff, K., et al. (1994) *EMBO J* **13**:4587-4596

43. Hug, H., et al. (1994) *FEBS Lett* **351**:311-313

44. Chinnaiyan, A.M., et al. (1996) *J Biol Chem* **271**:4961-4965

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Perl, Andras
Banki, Katalin

(ii) TITLE OF INVENTION: TRANSALDOLASE-MEDIATED REGULATION OF APOPTOSIS

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Jaeckle Fleischmann & Mugel, LLP
(B) STREET: 39 State Street
(C) CITY: Rochester
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 14614-1310

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/032,974
(B) FILING DATE: 13-DEC-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Braman, Susan J
(B) REGISTRATION NUMBER: 34,103
(C) REFERENCE/DOCKET NUMBER: 87687.97R457

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 716-262-3640
(B) TELEFAX: 716-262-4133

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1332 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(x) PUBLICATION INFORMATION:
(A) AUTHORS: Banki, K.
Halladay, D.
Perl, A.
(B) TITLE: Cloning and expression of the human gene for transaldolase. A novel highly repetitive element constitutes an integral part of the coding sequence.

(C) JOURNAL: J. Biol. Chem.
(D) VOLUME: 269
(E) ISSUE: 4
(F) PAGES: 2847-2851
(G) DATE: 1994

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCCGCG CCCGTCCCGT CGCCGCCGCC GCCGCCGCAG ACCCCTCGGT CTTGCTATGT
60

CGAGCTCACC CGTGAAGCGT CAGAGGATGG AGTCCGCGCT GGACCAGCTC AAGCAGTTCA
120

CCACCGTGGT GGCCGACACG GGCGACTTCC ACGCCATCGA CGAGTACAAG CCCCAGGATG
180

CTACCACCAA CCCGTCCCTG ATCCTGGCCG CAGCACAGAT GCCCGCTTAC CAGGAGCTGG
240

TGGAGGAGGC GATTGCCTAT GGCCGGAAGC TGGGCGGGTC ACAAGAGGAC CAGATTAAAA
300

ATGCTATTGA TAAACTTTTT GTGTTGTTTG GAGCAGAAAT ACTAAAGAAG ATTCCGGGCC
360

GAGTATCCAC AGAAGTAGAC GCAAGGCTCT CCTTTGATAA AGATGCGATG GTGGCCAGAG
420

CCAGGCGGCT CATCGAGCTC TACAAGGAAG CTGGGATCAG CAAGGACCGA ATTCTTATAA
480

AGCTGTCATC AACCTGGGAA GGAATTCAGG CTGGAAAGGA GCTCGAGGAG CAGCACGGCA
540

TCCACTGCAA CATGACGTTA CTCTTCTCCT TCGCCCAGGC TGTGGCCTGT GCCGAGGCGG
600

GTGTGACCCT CATCTCCCCA TTTGTTGGGC GCATCCTTGA TTGGCATGTG GCAAACACCG
660

ACAAGAAATC CTATGAGCCC CTGGAAGACC CTGGGGTAAA GAGTGTCACT AAAATCTACA
720

ACTACTACAA GAAGTTTAGC TACAAAACCA TTGTCATGGG CGCCTCCTTC CGCAACACGG
780

GCGAGATCAA AGCACTGGCC GGCTGTGACT TCCTCACCAT CTCACCCAAG CTCCTGGGAG
840

AGCTGCTGCA GGACAACGCC AAGCTGGTGC CTGTGCTCTC AGCCAAGGCG GCCCAAGCCA
900

GTGACCTGGA AAAAATCCAC CTGGATGAGA AGTCTTTCCG TTGGTTGCAC AACGAGGACC
960

AGATGGCTGT GGAGAAGCTC TCTGACGGGA TCCGCAAGTT TGCCGCTGAT GCAGTGAAGC
1020

TGGAGCGGAT GCTGACAGAA CGAATGTTCA ATGCAGAGAA TGGAAAGTAG CGCATCCCTG
1080

AGGCTGGACT CCAGATCTGC ACCGCCGGCC AGCTGGGATC TGACTGCACG TGGCTTCTGA
1140

TGAATCTTGC GTTTTTTACA AATTGGAGCA GGGACAGATC ATAGATTTCT GATTTTATGT
1200

AAAATTTTGC CTAATACATT AAAGCAGTCA CTTTTCCTGT GCTGTTTCAA AAAAAAAAAA
1260

AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
1320

AAAAAGGAAT TC
1332

What Is Claimed Is:

1. A method of regulating apoptosis of a cell, the method comprising increasing or decreasing levels of transaldolase protein enzymatic activity in the cell.

2. The method of claim 1 wherein levels of transaldolase protein enzymatic activity in the cell are increased to increase cell apoptosis.

3. The method of claim 2 wherein increasing levels of transaldolase protein enzymatic activity comprises increasing transaldolase gene expression of the transaldolase protein in the cell.

4. The method of claim 3 wherein increasing transaldolase gene expression comprises:
introducing a nucleic acid molecule encoding the transaldolase protein into the cell; and
allowing the cell to express the nucleic acid molecule resulting in the production of the transaldolase protein in the cell.

5. The method of claim 2 wherein increasing levels of transaldolase protein enzymatic activity comprises exposing the cell to a stimulant of the transaldolase protein.

6. The method of claim 1 wherein levels of transaldolase protein enzymatic activity in the cell are decreased to decrease cell apoptosis.

7. The method of claim 6 wherein decreasing levels of transaldolase protein enzymatic activity comprises decreasing transaldolase gene expression of the transaldolase protein in the cell.

8. The method of claim 7 wherein decreasing transaldolase gene expression comprises exposing the cell to a compound which decreases transaldolase gene expression of the transaldolase protein.

9. The method of claim 8 wherein the compound is an antisense nucleic acid molecule targeted to the transaldolase gene.

10. The method of claim 9 wherein exposing the cell to the antisense nucleic acid molecule comprises introducing the antisense nucleic acid molecule targeted to the transaldolase protein into the cell.

11. The method of claim 6 wherein decreasing levels of transaldolase protein enzymatic activity comprises exposing the cell to an inhibitor of the transaldolase protein.

12. A method of treating or preventing an abnormal condition in a subject resulting from abnormal cell apoptosis, the method comprising increasing or decreasing levels of transaldolase protein enzymatic activity in the cell of the subject.

13. The method of claim 12 wherein levels of transaldolase protein enzymatic activity in the cell are increased to increase cell apoptosis.

14. The method of claim 13 wherein increasing levels of transaldolase protein enzymatic activity comprises increasing transaldolase gene expression of the transaldolase protein in the cell.

15. The method of claim 14 wherein increasing transaldolase gene expression comprises:

introducing a nucleic acid molecule encoding the transaldolase protein into the cell; and

allowing the cell to express the nucleic acid molecule resulting in the production of the transaldolase protein in the cell.

16. The method of claim 13 wherein increasing levels of transaldolase protein enzymatic activity comprises exposing the cell to a stimulant of the transaldolase protein.

17. The method of claim 13 wherein the cell is selected from the group consisting of a cancer cell, a cell of a subject having an autoimmune disorder, and a cell of a subject having a viral infection.

18. The method of claim 12 wherein levels of transaldolase protein enzymatic activity in the cell are decreased to decrease cell apoptosis.

19. The method of claim 18 wherein decreasing levels of transaldolase protein enzymatic activity comprises decreasing transaldolase gene expression of the transaldolase protein in the cell.

20. The method of claim 19 wherein decreasing transaldolase gene expression comprises exposing the cell to a compound which decreases transaldolase gene expression of the transaldolase protein.

21. The method of claim 20 wherein the compound is an antisense nucleic acid molecule targeted to the transaldolase gene.

22. The method of claim 21 wherein exposing the cell to the antisense nucleic acid molecule comprises

introducing the antisense nucleic acid molecule targeted to the transaldolase protein into the cell.

23. The method of claim 18 wherein decreasing levels of transaldolase protein enzymatic activity comprises exposing the cell to an inhibitor of the transaldolase protein.

24. The method of claim 18 wherein the cell is selected from the group consisting of a cell of a subject having acquired immune deficiency syndrome, a cell of a subject having a demyelinating disease, a cell of a subject having a neurodegenerative disorder, a cell of a subject having a myelodysplastic syndrome, and a cell of a subject having a toxin-induced liver disease.

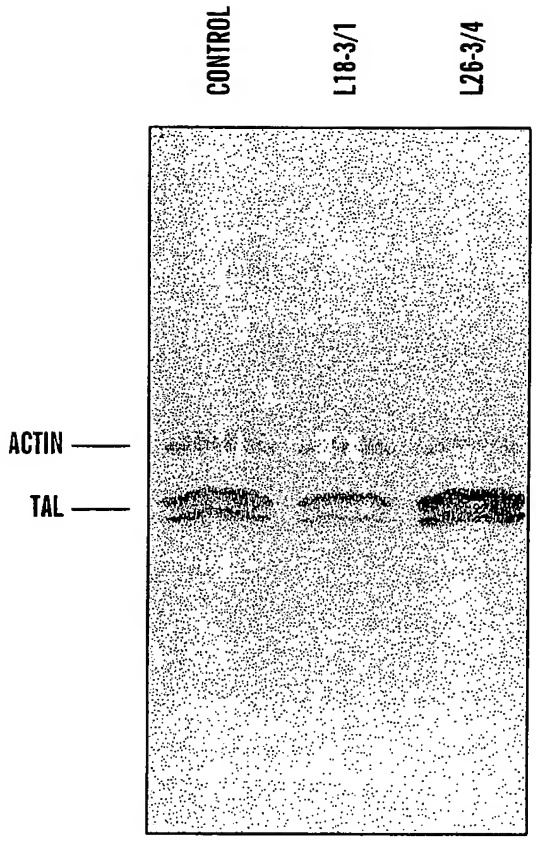


FIG. 1

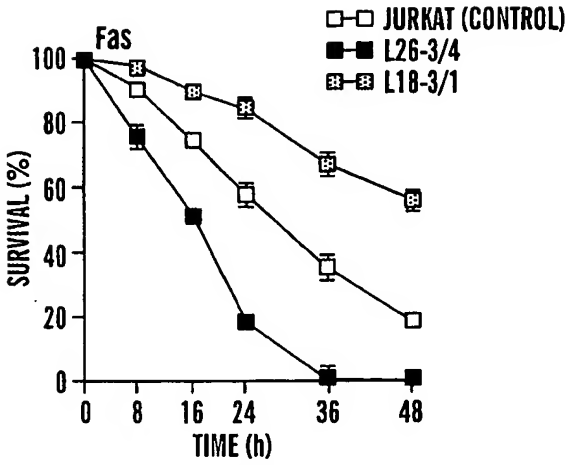


FIG. 2A

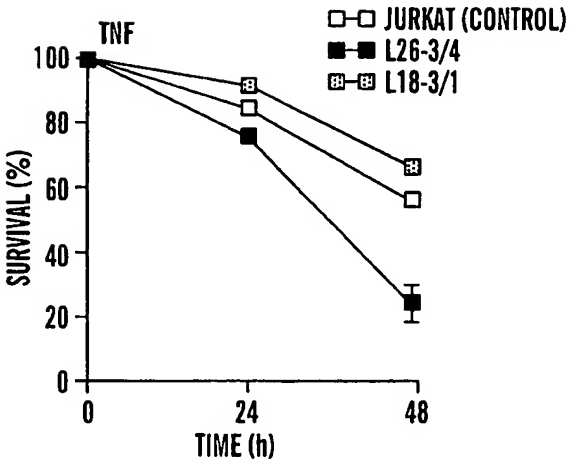


FIG. 2B

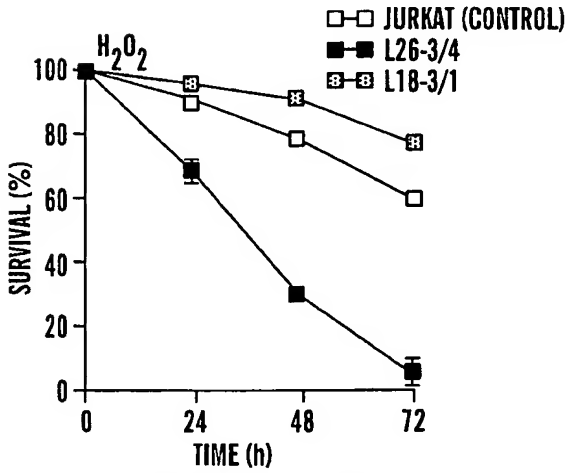


FIG. 2C

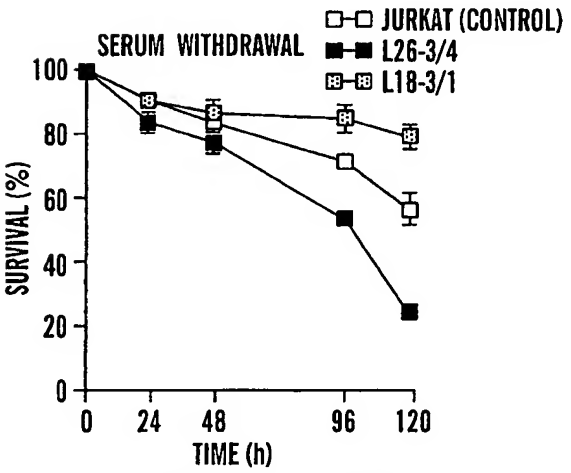


FIG. 2D

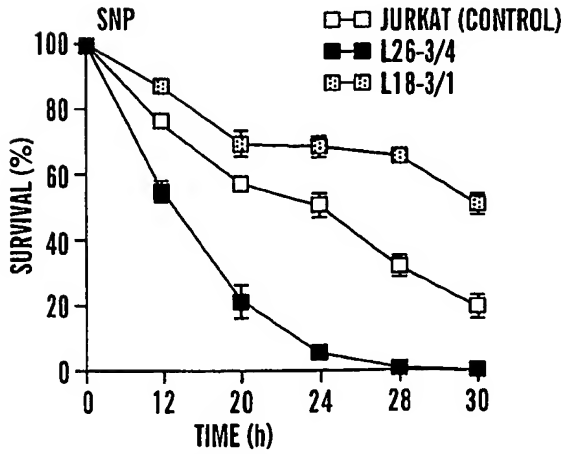


FIG. 2E

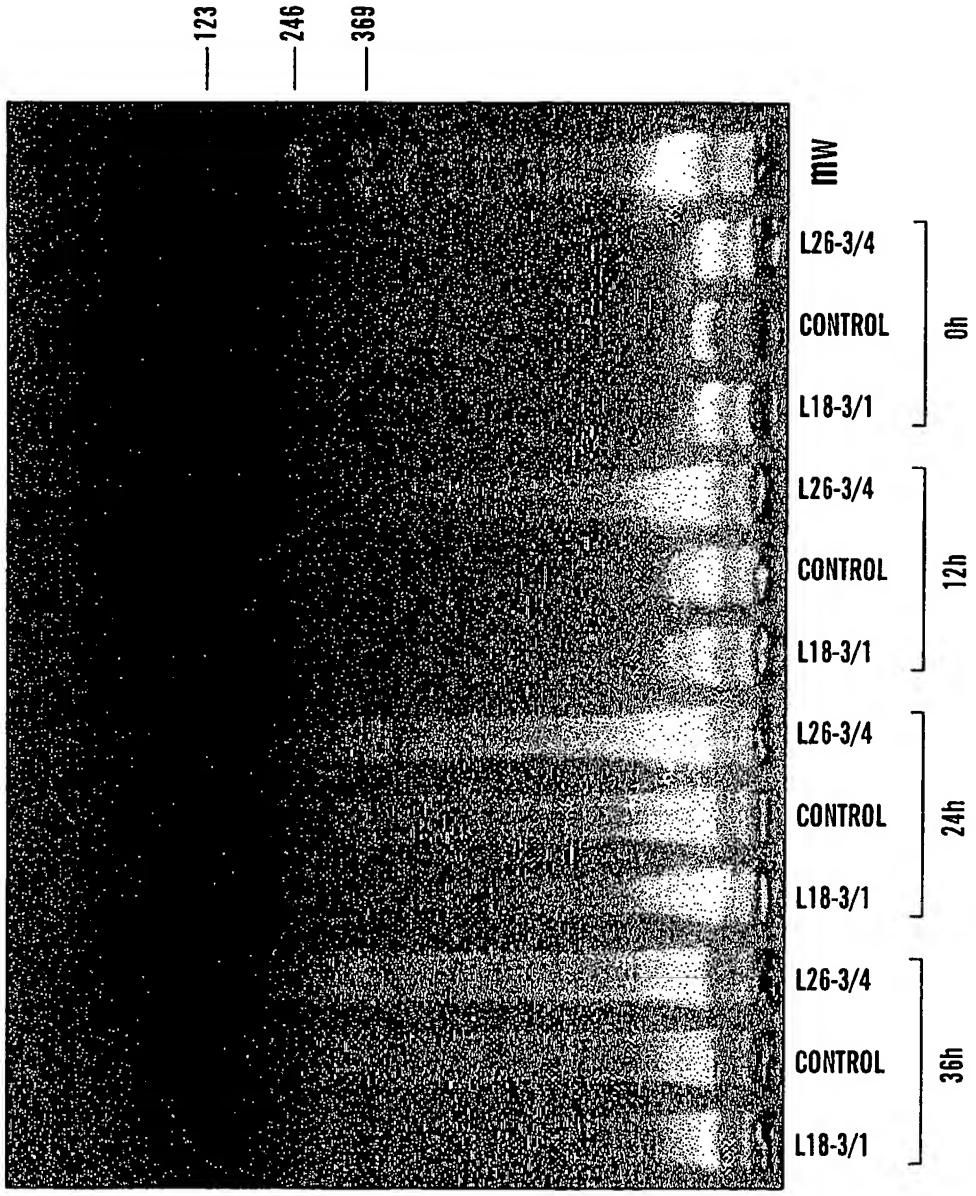

123
246
369
MW
L26-3/4
CONTROL
L18-3/1
0h
L26-3/4
CONTROL
L18-3/1
12h
L26-3/4
CONTROL
L18-3/1
24h
L26-3/4
CONTROL
L18-3/1
36h

FIG. 3

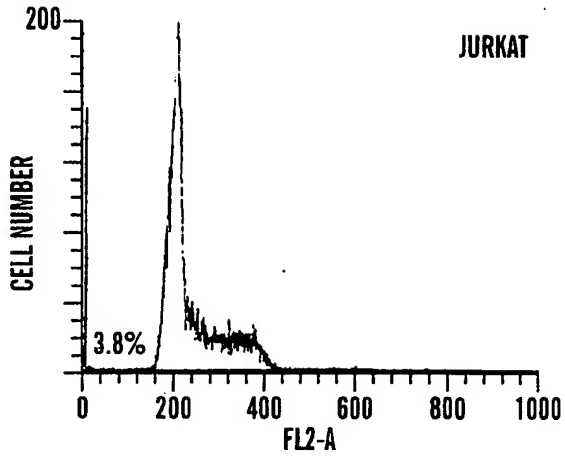


FIG. 4A

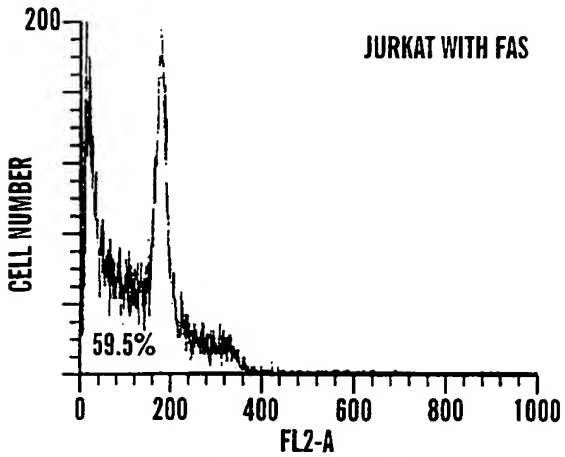


FIG. 4B

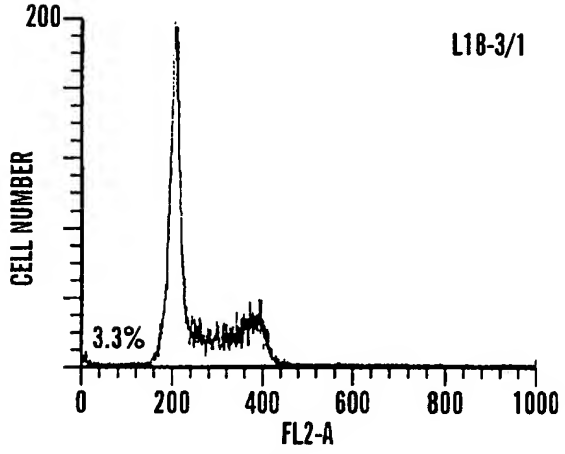


FIG. 4C

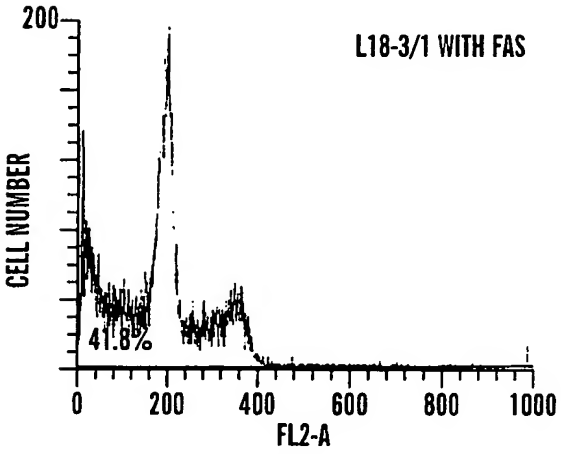


FIG. 4D

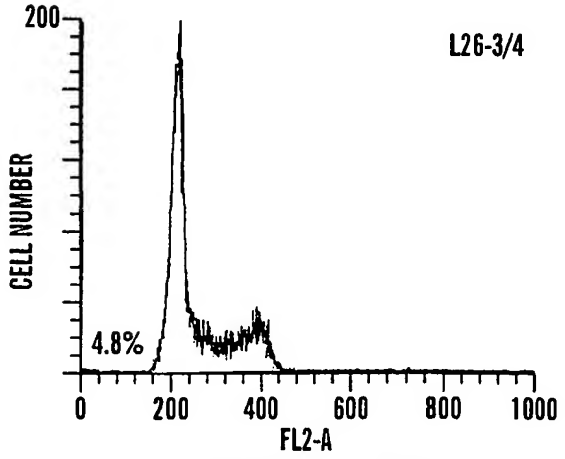


FIG. 4E

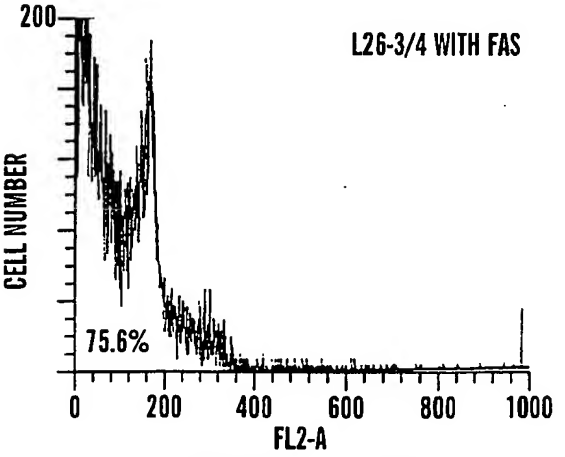


FIG. 4F

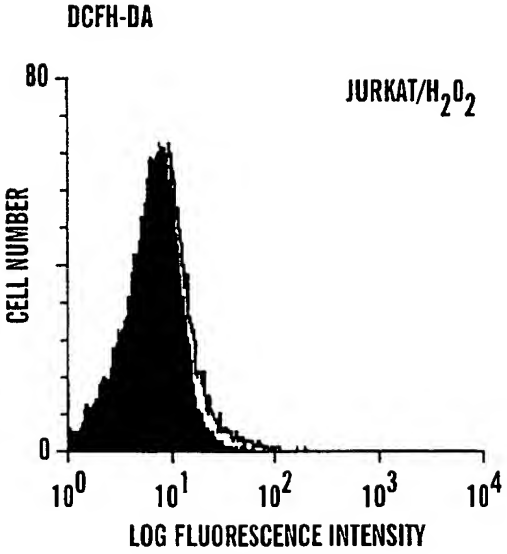


FIG. 5A

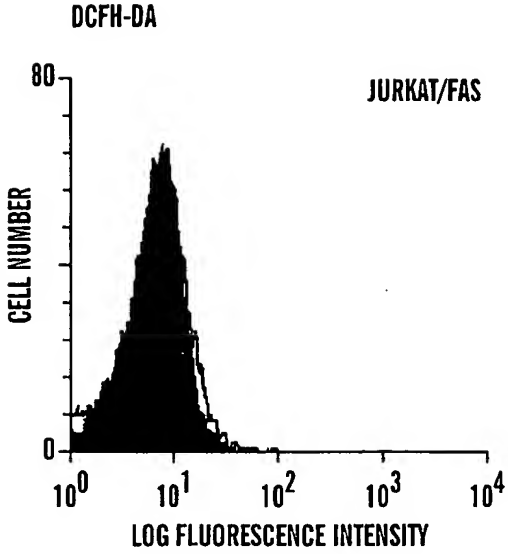


FIG. 5B

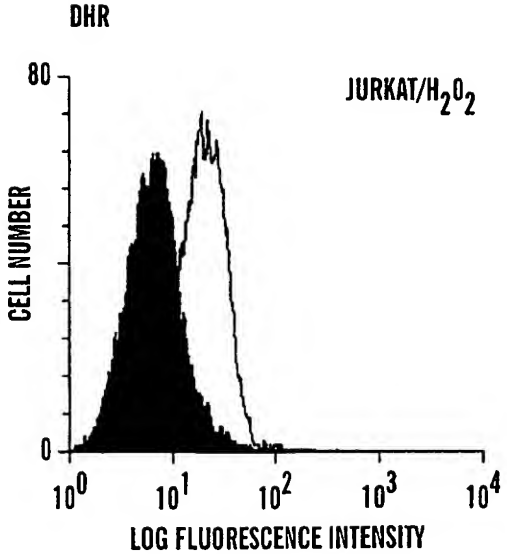


FIG. 5C

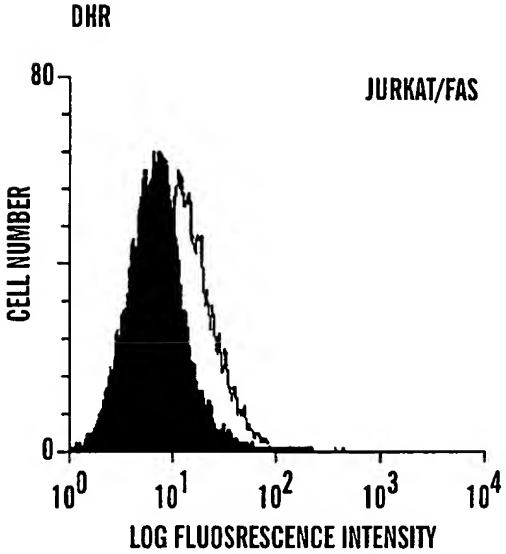


FIG. 5D

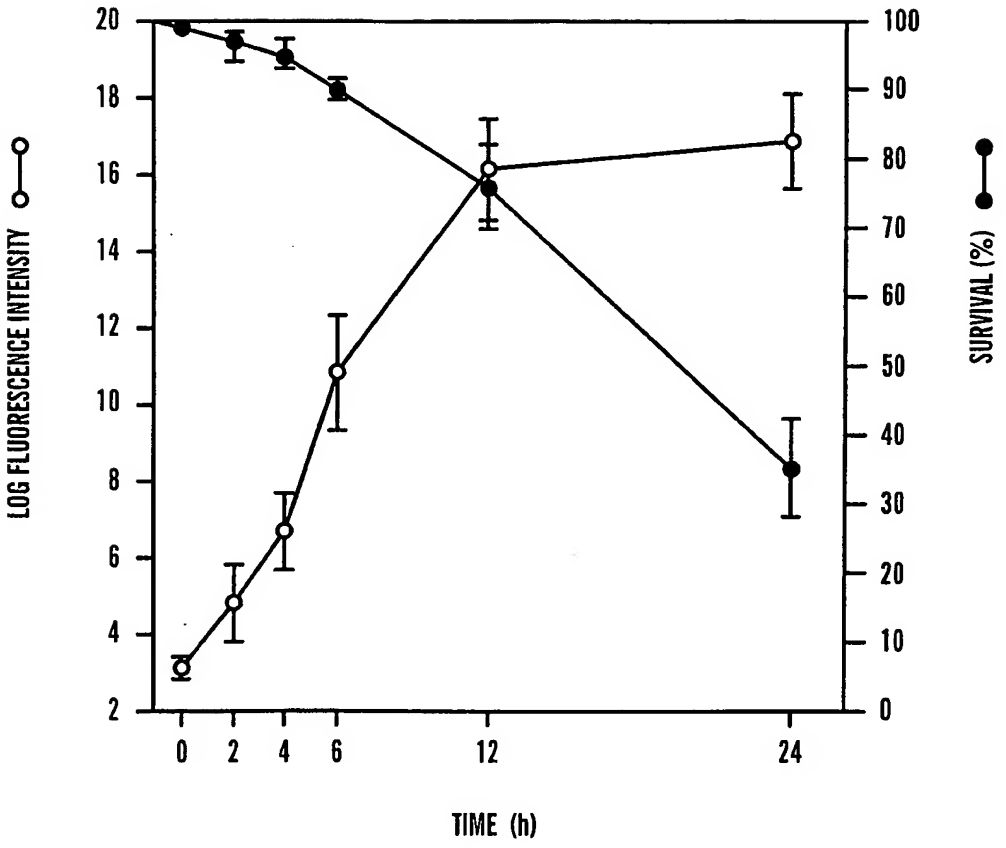

LOG FLUORESCENCE INTENSITY
SURVIVAL (%)
TIME (h)
20
18
16
14
12
10
8
6
4
2
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
12
24

FIG. 6

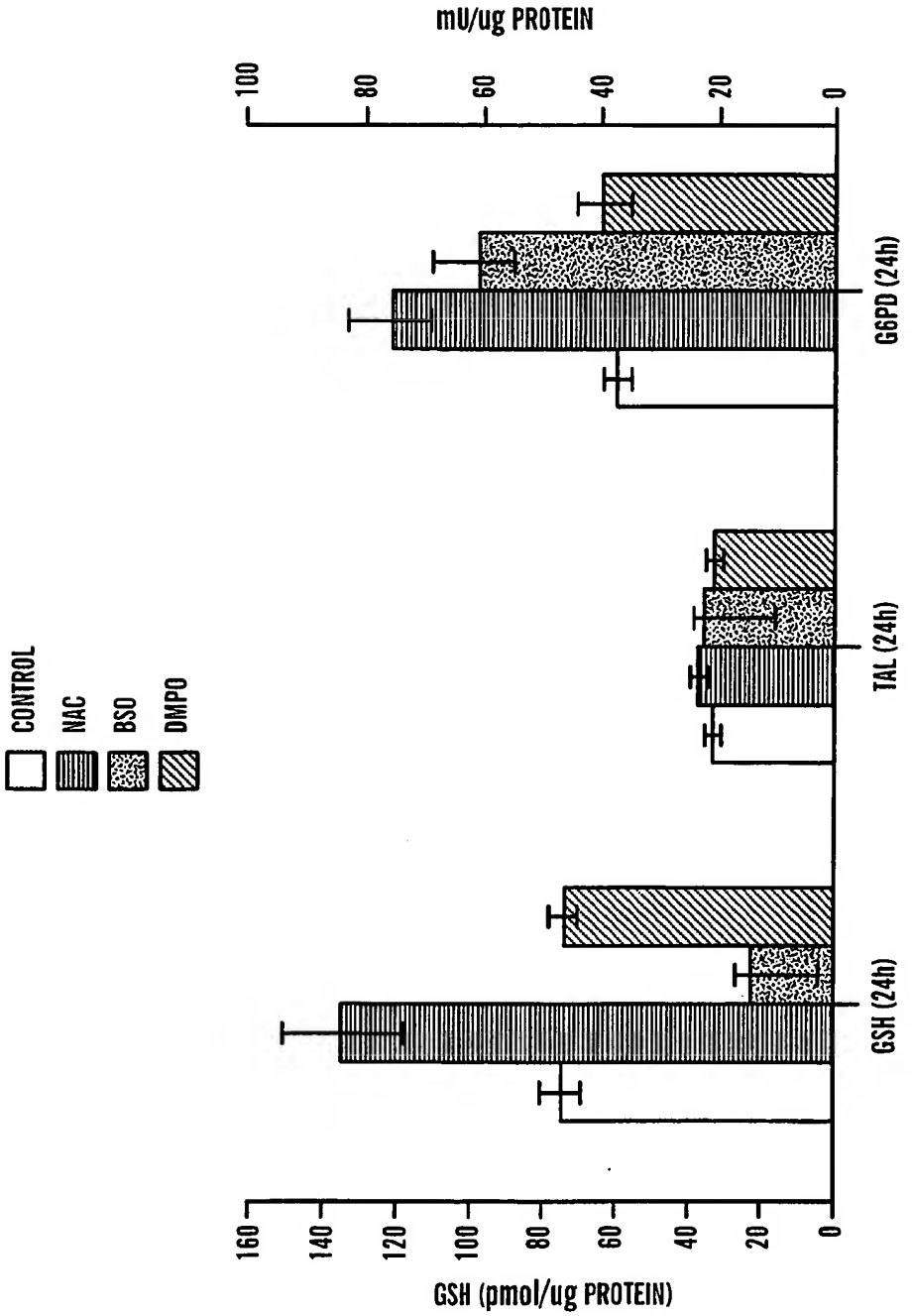

mU/ug PROTEIN
100
80
60
40
20
0
G6PD (24h)
TAL (24h)
GSH (24h)
CONTROL
NAC
BSO
DMPO
160
140
120
100
80
60
40
20
0
GSH (pmol/ug PROTEIN)

FIG. 7

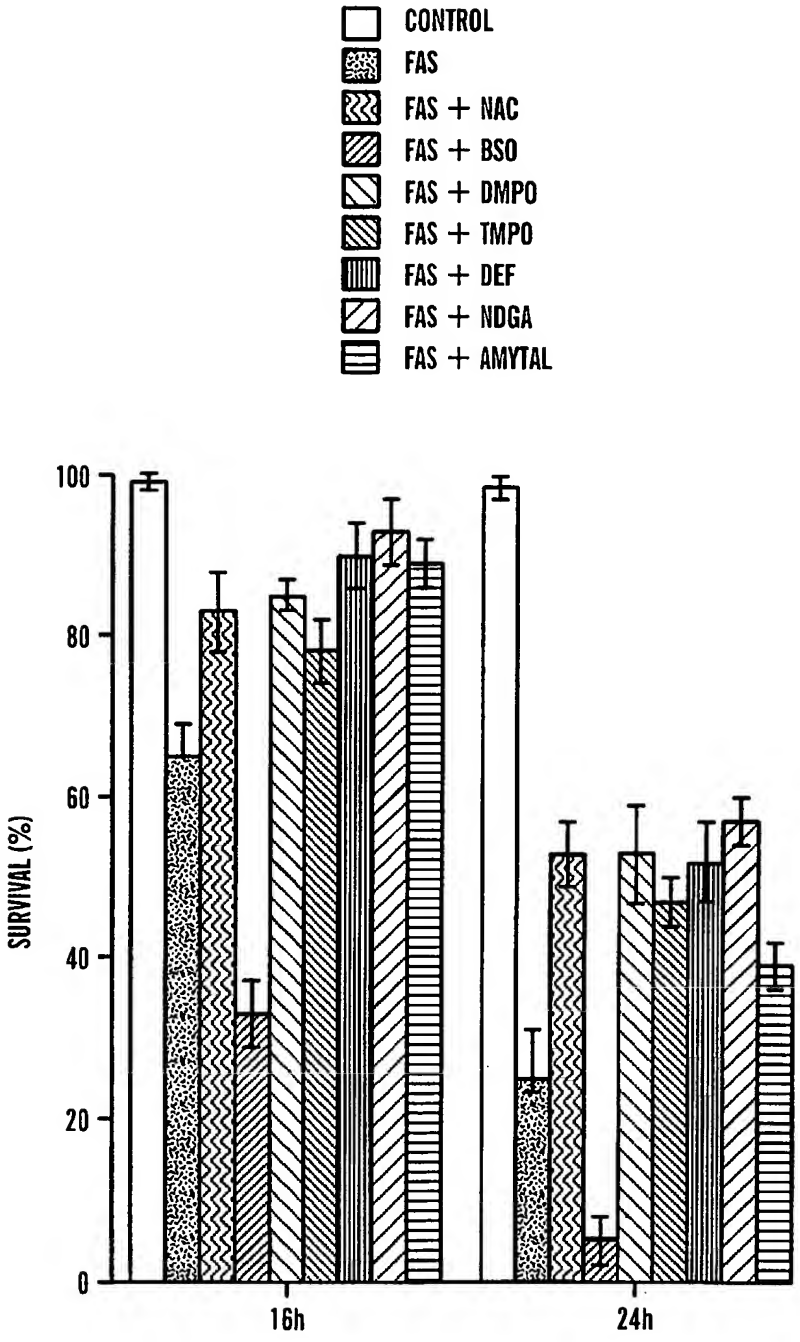

CONTROL
FAS
FAS + NAC
FAS + BSO
FAS + DMPO
FAS + TMPO
FAS + DEF
FAS + NDGA
FAS + AMYTAL
SURVIVAL (%)
100
80
60
40
20
0
16h
24h

FIG. 8

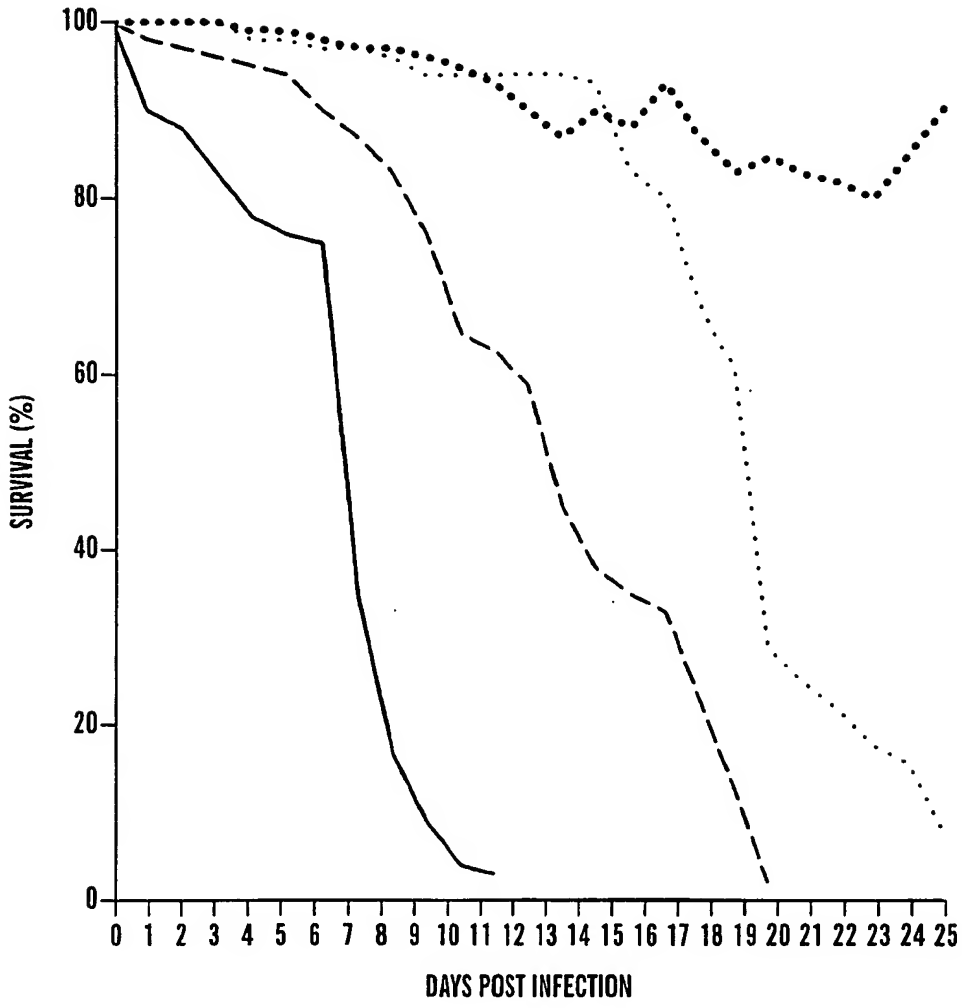

100
80
60
40
20
0
SURVIVAL (%)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
DAYS POST INFECTION

FIG. 9

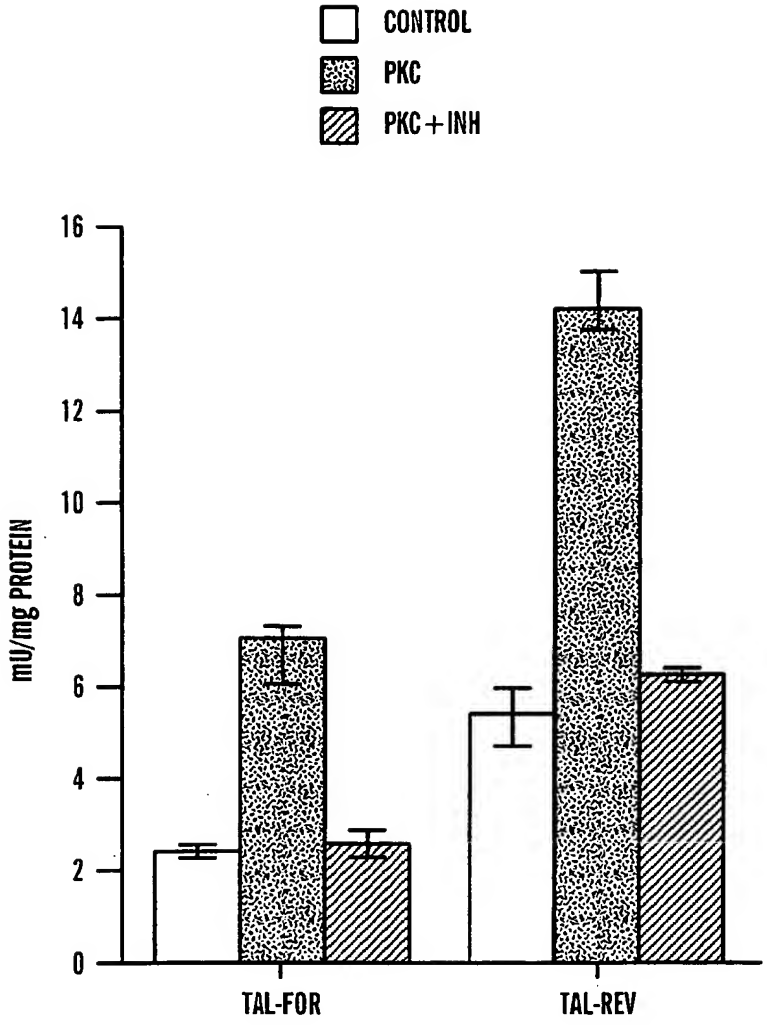

CONTROL
PKC
PKC + INH
16
14
12
10
8
6
4
2
0
mU/mg PROTEIN
TAL-FOR
TAL-REV

FIG. 10

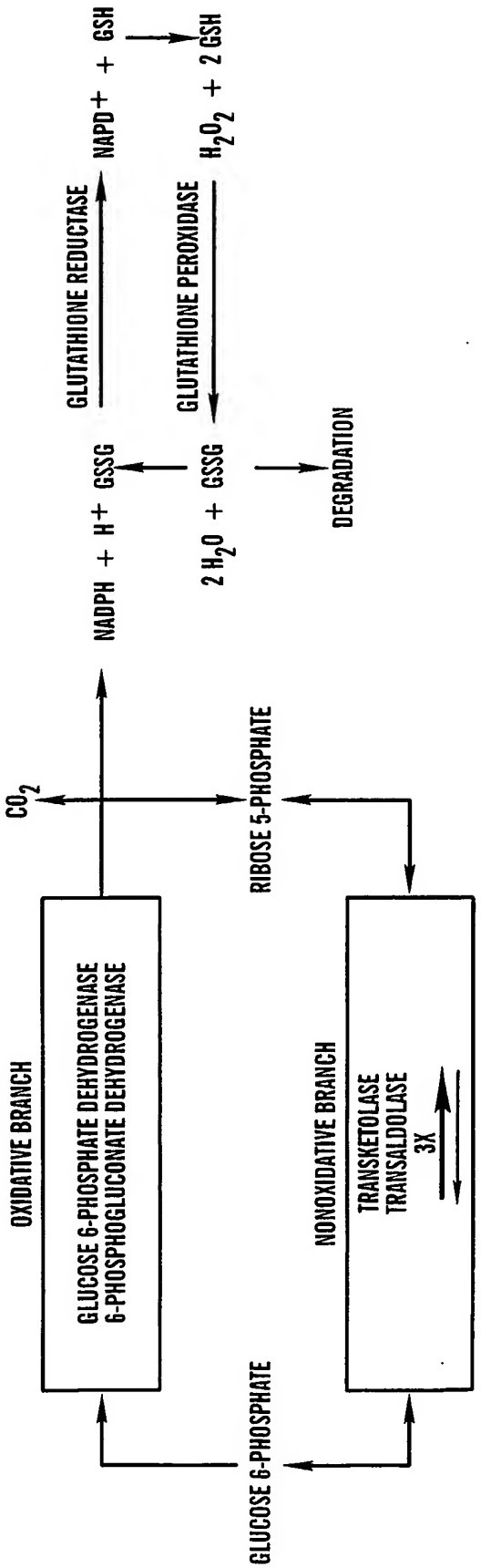

GLUCOSE 6-PHOSPHATE
OXIDATIVE BRANCH
GLUCOSE 6-PHOSPHATE DEHYDROGENASE
6-PHOSPHOGLUCONATE DEHYDROGENASE
NONOXIDATIVE BRANCH
TRANSKETOLASE
TRANSALDOLASE
3X
RIBOSE 5-PHOSPHATE
CO_2
NADPH + H^+
GSSG
$2\ H_2O$ + GSSG
DEGRADATION
GLUTATHIONE REDUCTASE
GLUTATHIONE PEROXIDASE
NAPD+ + GSH
H_2O_2 + 2 GSH

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22770

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 31/70; C12N 9/10, 15/12, 15/54
US CL : 435/69.1, 193, 375; 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/15, 69.1, 172.1, 172.3, 193, 375; 514/44; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN (BIOSIS, CAPLUS, LIFESCI, MEDLINE, INPADOC, WPIDS).
Search Terms: transaldolase, TAL, apoptosis, antisense, inhibit(ion), cell, cell death, Perl, Banki.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	BANKI et al. Glutathione Levels and Sensitivity to Apoptosisare Regulated by Changes in Transaldolase Expression. The Journal of Biological Chemistry, 20 December 1996, Vol. 271, No. 51, pages 32994-33001, see entire document.	1-24
X	EP 06202781 A1 (ONO PHARMACEUTICAL CO., LTD) 19 October 1994, see entire document.	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
26 FEBRUARY 1998

Date of mailing of the international search report
08 APR 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
THOMAS G. LARSON, PH.D.
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*